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SYSTEMS

Living Image® Software Version 3.0

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Living Image® Software

User's Manual

Version 3.0

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1 Welcome

What's New In the Living Image Software? 1
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The Living Image® software controls image acquisition on the IVIS® Imaging System and provides tools for optimizing image display and analyzing image data.

1.1 What's New In the Living Image Software?

The Living Image 3.0 software provides many new and improved features for all IVIS Systems. Table 1.1 lists the new Living Image software features and their availability on the different IVIS Imaging Systems.

Table 1.1 Living Image software

New Features	See Page	IVIS Imaging System					
		50	Lumina	100	200	Spectrum	3D
Spectral unmixing tools eliminate autofluorescence.	99					✓	
Fluorescence tomography algorithm (FLIT) enables 3D reconstruction of fluorescent sources.	118					✓	
Comprehensive image analysis and display tools that are organized in a convenient tool palette.	34	✓	✓	✓	✓	✓	✓
New visualization tools including an opacity slider, min/max color bar sliders, zoom, and panning tools.	45	✓	✓	✓	✓	✓	✓
Ability to *rebin* (increase or decrease the pixel size in an image) and smooth images.	47	✓	✓	✓	✓	✓	✓
Extensive selection of ROI (region of interest) tools for selecting and quantifying features or exporting data to other applications such as Microsoft Excel. Circle, rectangle, contour, and free-draw ROI shapes are available in manual or auto modes.	61	✓	✓	✓	✓	✓	✓
New image analysis features: line profiles, histograms, distance measurement, and pixel measurement.	48	✓	✓	✓	✓	✓	✓
Export image data in DICOM format for viewing with other DICOM readers.	29	✓	✓	✓	✓	✓	✓
Improved algorithms for fluorescent background correction and new Image Math tools automate background fluorescence subtraction.	79	✓	✓	✓	✓	✓	✓
New overlay tool that enables you to overlay two or more images using different color representations for displaying data from multiple reporters.	82	✓	✓	✓	✓	✓	✓
New acquisition sequence editor that is highly intuitive and supports in-place editing of sequence parameters.	23	✓	✓	✓	✓	✓	✓
New universal label set helps you label images consistently and in a manner that is compatible with database search engines.	24	✓	✓	✓	✓	✓	✓

Table 1.1 Living Image software

New Features	See Page	IVIS Imaging System					
		50	Lumina	100	200	Spectrum	3D
Faster, more robust "stop" feature for terminating image acquisition when you want to change imaging parameters and start over.		✓	✓	✓	✓	✓	✓
Biotrue Collaborative Data Management System, an optional web-based system for storing, managing, and sharing data	147	✓	✓	✓	✓	✓	✓
Improved planar spectral imaging feature for estimating depth of a luminescent source based on spectral information.	85				✓	✓	✓
Structured light analysis algorithm for determining the surface shape of the subject animal.	109				✓	✓	✓
The Diffuse Luminescent Imaging Tomography (DLIT) analysis algorithm for quantifying the intensity and location of bioluminescent sources in 3D space.	109				✓	✓	✓
A tissue database that contains wavelength-dependent absorption and scattering properties. A spectral database that includes emission spectra for several different luciferases.	115				✓	✓	✓
A digital Xenogen Mouse Atlas (male and female nude mouse) and the ability to automatically coregister the atlas with your DLIT 3D data. Import a custom organ atlas.	137				✓	✓	✓
Ability to import Open Inventor® files of segmented CT or MRI images and coregistering with a DLIT 3D image using a non-rigid registration algorithm.	140				✓	✓	✓
Create and export movies of tomographic data from multiple perspectives.	145				✓	✓	✓
Reconfigured menu bar that matches the standard layout of Windows/Macintosh applications.		✓	✓	✓	✓	✓	✓
Living Image software remembers the path of recently accessed folders and provides auto-save capability during imaging.		✓	✓	✓	✓	✓	✓
Integrated instrument control, image acquisition and analysis tools written in C++ for enhanced stability and performance.		✓	✓	✓	✓	✓	✓
Extensive help tools and PDF manual with quick links for rapidly finding information.		✓	✓	✓	✓	✓	✓

1.2 About This Manual

This user manual explains how to acquire images on an IVIS Imaging System and analyze image data. The manual provides detailed instructions and screenshots that depict the system response.

NOTE

Sometimes the screenshots in the manual may not exactly match those displayed on your screen.

For more details on the IVIS Imaging System, please see the appropriate *IVIS Imaging System Hardware Manual.*

Conventions Used In the Manual

Convention	Example
Menu commands are bolded.	To open image data, select **File →Open Dataset** on the main bar.
Toolbar button names are bolded.	To open image data, click the **Open Dataset** button .
Numbered steps explain how to carry out a procedure.	1. To start the Living Image software, click the icon on the desktop.
A dash (—) precedes the description of the system response to a procedure.	— The main window appears.
Document names are italicized.	*Living Image Software User's Guide*
Note information	**NOTE** A note presents pertinent details on a topic.
Caution information	**CAUTION** **CAUTION!** A caution note warns you that your actions may have nonreversible consequences or may cause loss of data.
Important information	**IMPORTANT** **ALERT!** Important information advises you of actions that are essential to the correct performance of the instrument or software.

1.3 Contacting Xenogen Technical Support

If you need technical support, please contact Xenogen at:

Telephone:	1.888.810.8055 (Toll Free in the United States) 1.508.435.9761
E-mail:	tech.support@caliperls.com
Fax:	1.508.435.0950
Address:	Xenogen Corporation, A Division of Caliper Life Sciences 68 Elm Street Hopkinton, MA 01748 USA

2 Getting Started

Starting the Living Image® Software 5
Basic Living Image® Software Tasks 6
Living Image Help 7

For information on installing the software, see the Installation Guide included on the Living Image CD ROM. By default, the software is installed to:

PC: C:Programs:Xenogen:Living Image 3.0

Macintosh: Applications:Xenogen:LivingImage 3.0

2.1 Starting the Living Image® Software

All components of the IVIS® Imaging System should be left on at all times because of the long cooling time required to reach operating (*demand*) temperature. It is also important to leave the system on to enable automatic overnight electronic background measurements. Periodically rebooting the computer is permissible and does not affect the camera operation.

To start the software:

1. **PC Users:** Click the Windows **Start** menu button and select **All Programs →Living Image**. Alternatively, click the Living Image® software desktop icon.

   Macintosh Users: Click the Living Image software desktop icon or run the software from the application folder.

2. When prompted, select a user ID or enter a new User ID (up to three letters), and click **Done.**

   — The Living Image software user interface appears (Figure 2.1).

NOTE

The Living Image software on the PC workstation that controls the IVIS Imaging System includes both the acquisition and analysis features. The Living Image software on other workstations includes only the analysis features. Macintosh users have access to only the analysis features of the Living Image software.

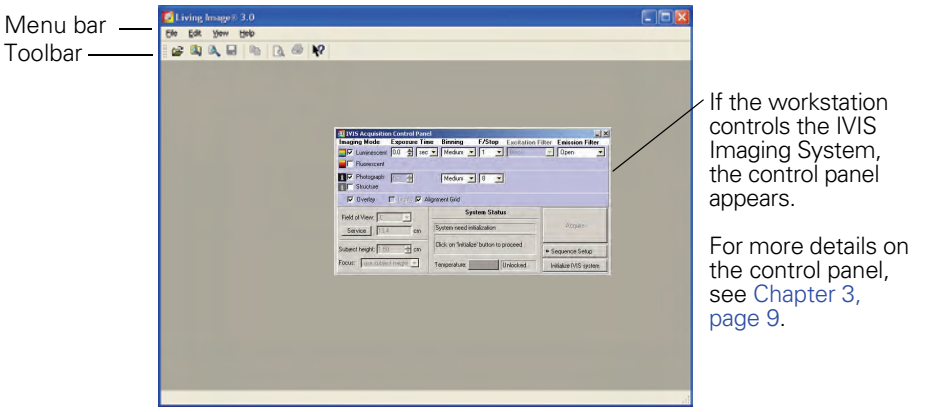
Menu bar
Toolbar
If the workstation controls the IVIS Imaging System, the control panel appears.
For more details on the control panel, see Chapter 3, page 9.

Figure 2.1 Living Image software user interface at startup
For more details on the menu bar and toolbar, see Appendix G, page 199.

2.2 Basic Living Image® Software Tasks

Here are links to some common tasks in the Living Image software.

Table 2.1 Basic Living Image software functions

Task	See Page
Acquire an image:	
IVIS® Lumina or Spectrum	18-19
IVIS 50, 100, or 200 Imaging System Series	18-19
IVIS 3D Imaging System Series	20-21
Acquire an image sequence:	23-24
Acquire an image sequence for bioluminescent 3D tomographic analysis:	
IVIS 200 Imaging System Series	110
IVIS 3D Imaging System Series	110
Acquire an image sequence for fluorescent 3D tomographic analysis (IVIS Spectrum only)	118
Save or export image data.	29
Browse and open image data.	31
Adjust the image display.	45
Correct or filter image data.	46
Make image measurements.	48
Draw a region of interest (ROI) on an image.	57
Mathematically combine images.	78
Overlay multiple images.	82
Remove tissue autofluorescence from a fluorescent image:	79
Using spectral unmixing (IVIS Spectrum only).	99
By subtracting a fluorescence background image.	79
Planar spectral image analysis.	88
Reconstruct 3D bioluminescent sources.	109

Table 2.1 Basic Living Image software functions

Task	See Page
Display organs on a 3D reconstruction.	137
Upload or download data from the Biotrue Collaborative Data Management System (an optional feature).	147

2.3 Living Image Help

There are several ways to obtain help on the software features:

- To view a tooltip about a button function, put the mouse cursor over the button.
- To view a brief description about an item in the user interface, click the toolbar button, then click the item.
- Press F1 or select **Help →User Guide** on the menu bar to display the Living Image 3.0 Software User's Manual (.pdf).

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3 Initializing the System & Acquiring Images

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Checking the System Temperature 10
Imaging Basics 10
About the Control Panel 12
Acquiring Images 17
Manual Focusing 28
Saving or Exporting Image Data 29

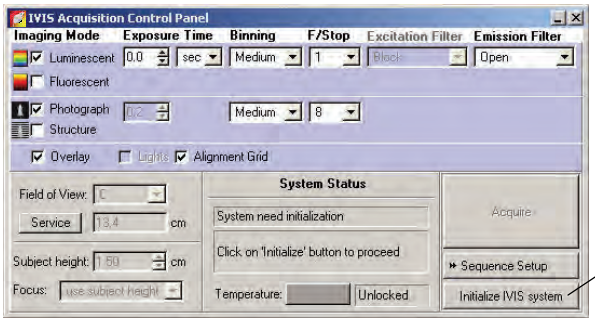
The IVIS® System is ready for image acquisition after the system is initialized and the demand (operating) temperature is locked.

NOTE

The Living Image® software on the PC workstation that controls the IVIS Imaging System includes both the acquisition and analysis features. The Living Image software on other workstations includes only the analysis features.

3.1 Initializing the IVIS Imaging System

The imaging system must be initialized each time the Living Image® software is started or if the power has been cycled to the imaging chamber or the camera controller (a component of some IVIS systems). The initialization procedure moves every motor-driven component in the system (for example, stage and lens) to a home position, resets all electronics and controllers, and restores all software variables to the default settings. Initialization may be useful in error situations. For further details on instrument operation, see the hardware manual for your IVIS Imaging System.



To initialize the system:

1. Start the Living Image software. (For more details, see Chapter 2, page 5.)
2. In the control panel, click **Initialize IVIS System**.
   - You will hear the motors move.

Figure 3.1 Control panel, initializing the IVIS Imaging System.
The control panel is only available on the PC workstation that controls the imaging system.

NOTE

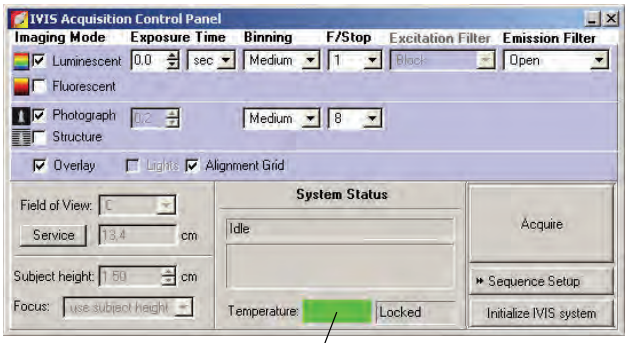
The items available in the IVIS System control panel depend on the particular IVIS Imaging System and the imaging mode selected (luminescent or fluorescent, Image Setup or Sequence Setup mode).

3.2 Checking the System Temperature

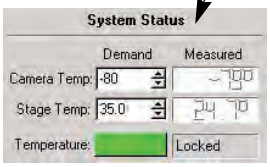
The temperature box in the IVIS System control panel indicates the temperature status of the charge coupled device (CCD) camera (Figure 3.2). At startup, the temperature box is red and turns green when the temperature is locked at the demand temperature (-90° C or -105° C for IVIS Systems cooled by a Cryotiger® unit), indicating the instrument is ready for operation.

The demand temperature for the CCD camera is fixed. Electronic feedback control maintains the CCD camera temperature to within a few degrees of the demand temperature.

The default stage temperature on the IVIS® Imaging System 200 Series is 37° C, but may be set to a temperature from 25-40° C.



Click the temperature box to display the demand and measured temperatures of the CCD camera and stage.



Temperature box color indicates:

- System not initialized.
- System is initialized, but CCD camera temperature is out of range.
- System is initialized and CCD camera is at or within acceptable range of the demand temperature and locked. The system is ready for imaging.

Figure 3.2 Checking the system temperature

3.3 Imaging Basics

For luminescent imaging, the system acquires two images:

- A photographic image - a short exposure of the subject illuminated by the lights located at the top of the imaging chamber (Figure 3.3). The photographic image is displayed as a grayscale image.
- A luminescent image - a longer exposure of the subject taken in darkness to capture low level luminescence emission. The luminescent image is displayed in pseudocolor that represents intensity.

The Living Image software automatically coregisters the luminescent and photographic image to generate an *overlay* image (Figure 3.4).

For IVIS Imaging Systems with fluorescent imaging capability, a photographic and fluorescent image can be acquired and coregistered. For more information on fluorescent imaging, see Appendix D, page 165.

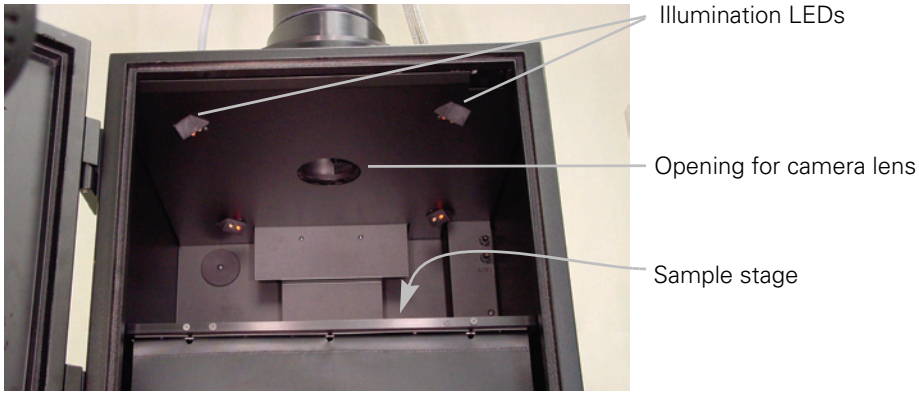
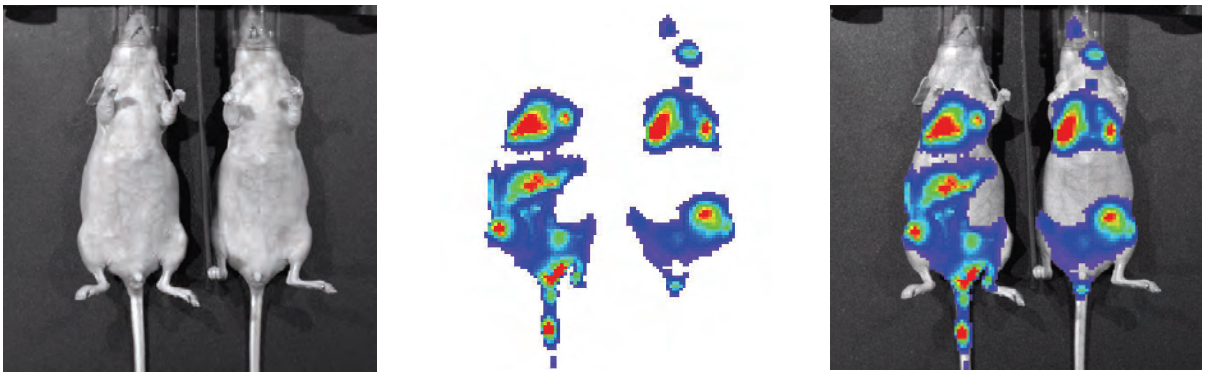


Figure 3.3 IVIS® Imaging System 100 Series, interior view.



Photographic image | Luminescent image | Overlay image

Figure 3.4 Image types
The software automatically coregisters the luminescent and photographic images to produce the overlay image.

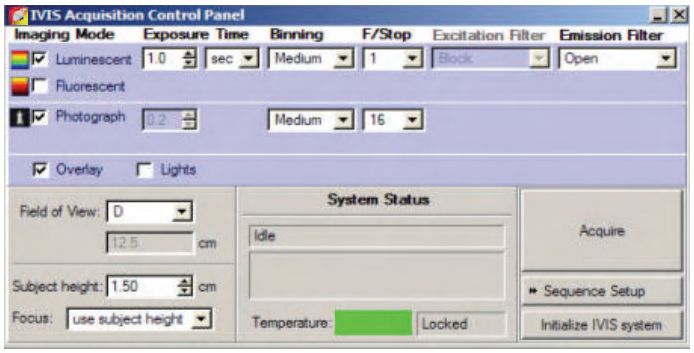
3.4 About the Control Panel

The IVIS System control panel provides the image acquisition functions (Figure 3.5).

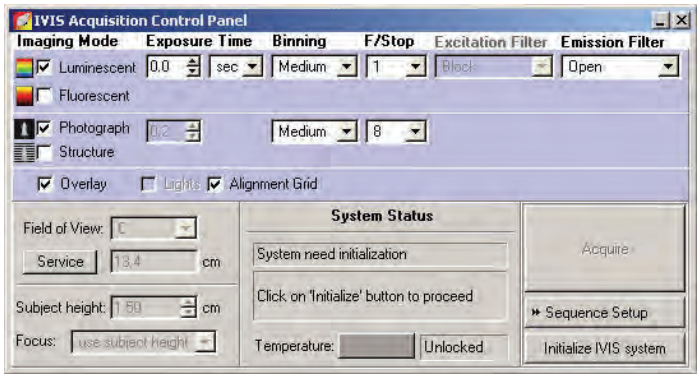
NOTE

The items available in the IVIS System control panel depend on the particular IVIS Imaging System and the imaging mode selected (luminescent or fluorescent, Image Setup or Sequence Setup mode).

IVIS Lumina and
IVIS Imaging System 100 Series

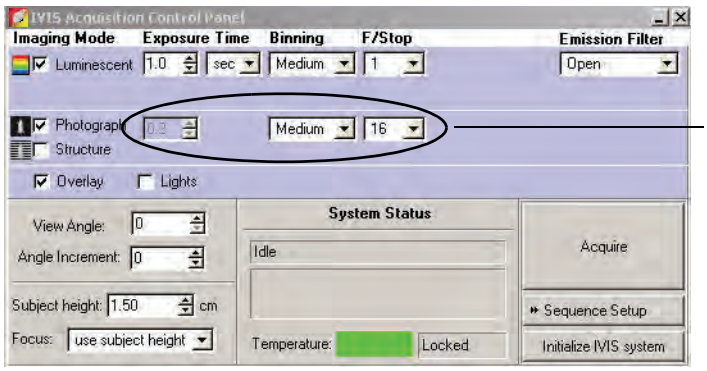


IVIS Spectrum and
IVIS Imaging System 200 Series



This row specifies settings for the luminescent or fluorescent image.

IVIS Imaging System 3D Series



This row specifies settings for the photographic image.

Figure 3.5 Control panel

Table 3.1 General controls for all IVIS Systems

Item in the Control Panel	Description
Luminescent	Choose this option to acquire a luminescent image.
Fluorescent	Choose this option to acquire a fluorescent image.
Exposure time	The length of time that the shutter is open during acquisition of a photographic or luminescent image. The luminescent or fluorescent signal level is directly proportional to the exposure time. The goal is to adjust the exposure time to produce a signal that is well above the noise (>600 counts recommended), but less than the CCD camera saturation of ~60,000 counts. Luminescent exposure time is measured in seconds or minutes. The minimum calibrated exposure time is 0.5 seconds. The exposure time for fluorescent images is limited to 60 seconds to prevent saturation of the CCD. There is no limit on the maximum exposure time for luminescent images; however, there is little benefit to exposure times greater than five minutes.The signal is linear with respect to exposure time over the range from 0.5 to 10 minutes. Integration times less than 0.5 seconds are not recommended due to the finite time required to open and close the lens shutter.
Binning	Controls the pixel size on the CCD camera. Increasing the binning increases the pixel size and the sensitivity, but reduces spatial resolution. Binning a luminescent image can significantly improve the signal-to-noise ratio. The loss of spatial resolution at high binning is often acceptable for *in vivo* images where light emission is diffuse. For more details on binning, see Appendix B, page 156. Recommended binning: 1-4 for imaging of cells or tissue sections, 4-8 for *in vivo* imaging of subjects, and 8-16 for *in vivo* imaging of subjects with very dim sources.
f/stop	Sets the size of the camera lens aperture.The aperture size controls the amount of light detected and the depth of field. A larger f/stop number corresponds to a smaller aperture size and results in lower sensitivity because less light is collected for the image. However, a smaller aperture usually results in better image sharpness and depth of field. A photographic image is taken with a small aperture (f/8 or f/16) to produce the sharpest image and a luminescent image is taken with a large aperture (f/1) to maximize sensitivity. For more details on f/stop, see Appendix B, page 155.
Excitation Filter	A drop-down list of fluorescence excitation filters. For fluorescent imaging, choose the appropriate filter for your application (GFP, DsRed, Cy5.5, or ICG). For bioluminescent imaging, **Block** is selected by default. If you select **Open**, no filter is present. For systems equipped with spectral imaging capability, choose the appropriate emission filter for your application (see Chapter 7, page 85). Note: The excitation filter selection automatically sets the emission filter position.
Emission Filter	A drop-down list of fluorescence emission filters located in front of the CCD lens. The emission filter wheel is equipped with filters for fluorescence or spectral imaging applications. The number of filter positions (6 to 24) depends on the system. For bioluminescent imaging, the **Open** position (no filter) is automatically selected by default.
Photographic	Choose this option to automatically acquire a photographic image. The illumination lights at the top of the imaging chamber are on during a photographic image so that the system can acquire a black and white photograph of the sample(s). Note: You can adjust the appearance of the photographic image using the Bright and Gamma controls (see *Photo Adjustment*, page 46).
Auto	If this option is chosen, the software automatically sets the exposure time to produce a good photographic image.
Fluor Lamp Level	Sets the illumination intensity level of the excitation lamp used in fluorescent imaging (Off, Low, High, and Inspect). The Low setting is approximately 18% of the High setting. Inspect turns on the QTH illumination lamp so that you can manually inspect the excitation lamp. Note: Make sure that the filters of interest are selected in the filter drop-down lists before you select Inspect. The Inspect operation automatically positions the selected filters in the system before turning on the lamp. Subsequent changes to the filter popup menus will have no effect until another Inspect operation is performed.
Overlay	If this option is chosen, the system automatically acquires a photographic image followed by a luminescent image, then coregisters the two images.
Lights On	Turns on the lights located at the top of the imaging chamber.

Table 3.1 General controls for all IVIS Systems

Item in the Control Panel	Description
Field of View (FOV)	Sets the size of the stage area to be imaged by adjusting the position of the stage and lens. The FOV is the width of the square area (cm) to be imaged. A smaller FOV gives a higher sensitivity measurement, so it is best to set the FOV no larger than necessary to accommodate the subject or area of interest. The FOV also affects the depth of field (range in which the subject is in focus). A smaller FOV results in a narrower depth of field. Select the FOV by choosing a setting from the drop-down list. For more details on the calibrated FOV positions A-E, see Table 3.5, page 16.
Service	Click to move the stage to a position for cleaning.
Load	Click to move the stage from the cleaning position back to the home position.
Subject height (cm)	Sets the position of the focal plane of the lens/CCD system by adjusting the stage position. The subject height is the distance above the stage that you are interested in imaging. For example, to image a mouse leg joint, set the subject height to a few mm. To image the uppermost dorsal side of a mouse, set the subject height to the 1.5 - 2.0 cm. The default subject height is 1.5 cm. **IMPORTANT** **ALERT!** The IVIS® System has a protection system to prevent instrument damage, however always pay close attention to subject height, particularly on the IVIS Imaging System 200 Series. For example, it is possible for a large subject (10 cm ventral-dorsal height) to contact the top of the imaging chamber if you set the subject height = 0 and choose a small FOV.
Focus	Drop-down list of focusing methods available: **Use subject height** - Choose this option to set the focal plane at the specified subject height. **Manual** - Choose this option to open the Focus Image window so that you can manually adjust the stage position. For more details on manual focusing, see Figure 3.16.
Temperature	The temperature box color indicates the temperature and status of the system: System not initialized. System initialized, but the CCD temperature is out of range. System is initialized and the CCD temperature is at or within acceptable range of the demand temperature and locked. The system is ready for imaging. Click the temperature box to display the actual and demand temperature of the CCD and stage. For more details, see page 10.
Acquire	Click to acquire an image using the settings and options selected in the control panel or to acquire an image sequence specified in the Sequential Setup table.
Sequence Setup	Click to display the Sequence Editor so that you can specify and manage sequence acquisition parameters or open sequence acquisition parameters (xsq). For more details on acquiring an image sequence, see page 23-24.
Image Setup	Click to close the Sequence Editor.
Initialize IVIS system	Click to initialize the IVIS Imaging System. For more details on initializing the system, see page 9.

Table 3.2 Additional IVIS® System Controls for the IVIS Imaging System 200 Series or IVIS Spectrum

Item in the Control Panel IVIS Imaging System 200 Series	Description
Alignment grid	Choose this option to activate a laser-generated alignment grid on the stage when the imaging chamber door is opened. The alignment grid is set to the size of the selected FOV. The grid automatically turns off after two minutes. If subject alignment is not completed in two minutes, place a check mark next to **Enable Alignment Grid** to turn on the grid. **Note:** The horizontal cross hair of the alignment grid is offset appropriately to take into account the height entered in the Subject height box.
Focus	**Scan Mid Image** - Choose this option in the Focus drop-down list to set the focal plane at the maximum dorso-ventral height of the subject at the middle of the animal. This focusing method uses the laser to scan horizontally across the middle of the subject to determine the maximum subject height along this line. This option is well suited for animal imaging because the peak height is clearly identified as the maximum height on the dorsal side along the mid-plane of the animal. **Note:** This focusing method is not recommended for microplates or when using a high magnification field of view (FOV A = 4.0 cm). In these situations, Manual or Subject Size focus methods are recommended.
Structure	Choose this option to take a structured light image (an image of parallel laser lines scanned across the subject) when you click **Acquire**. The structured light image is used to reconstruct the surface topography of the subject which is an input to the Diffuse Luminescence Imaging Tomography (DLIT™) algorithm that computes the 3D location and brightness of luminescent sources. When this option is chosen, the f/stop and exposure time are automatically set to defaults for the structured light image (f/8 and 0.2 sec, respectively). The spatial resolution of the computed surface depends on the line spacing of the structured light lines. The line spacing and binning are automatically set to the optimal values determined by the FOV (stage position) and are not user-modifiable.
Transillumination Setup (IVIS Spectrum only)	Choose this option to display the transillumination setup window that enables you to select the locations for image acquisition using bottom illumination that originates beneath the stage.

Table 3.3 Additional IVIS System controls for the IVIS Imaging System 3D Series

Item in the Control Panel IVIS Imaging System 3D Series	
Angle	The starting position of the CCD camera relative to the imaging stage. The first image in a sequence is acquired at this angle.
Inc	The number of degrees between each successive position of the CCD camera during the acquisition of an image sequence.
Structure	Choose this option to take a structured light image (an image of parallel lines scanned across the subject) when you click **Acquire**. For more details, see Structure in Table 3.2.

Table 3.4 Additional IVIS System controls for the IVIS Spectrum

Item in the Control Panel IVIS Imaging System 3D Series	
Transillumination	Choose this option to for bottom illumination excitation light.
Setup	Click to display the Transillumination setup window Figure 3.12.
Structure	Choose this option to take a structured light image (an image of parallel lines scanned across the subject) when you click **Acquire**. For more details, see Structure in Table 3.2.

Table 3.5 Typical field of view (FOV) settings

FOV Setting	IVIS Imaging System		
	Lumina	**100 Series**	**200 Series**
	FOV (cm)		
A	4	10	3.9
B	7	15	6.5
C	10	20	13
D	12	25	19.5
E			26

NOTE

There are no FOV settings for the IVIS® Imaging System 3D Series because the instrument acquires images from different angles at the same FOV.

3.5 Acquiring Images

To acquire an image or image sequence:

1. Start the Living Image software (double-click the icon on the desktop).
2. Initialize the IVIS System and confirm or wait for the CCD temperature to lock. (For more details, see Chapter 2, page 5.)

NOTE

Systems equipped with thermoelectric cooling may require several minutes to reach the demand temperature.

3. Place the anesthetized subject(s) in the imaging chamber and close the door. (For details on anesthetizing animals, see the *Xenogen XGI-8 Gas Anesthesia System User Manual*.)
4. Acquire the image or image sequence. Table 3.6 shows where to find details on image acquisition for your IVIS system and imaging mode.

Table 3.6 Image acquisition

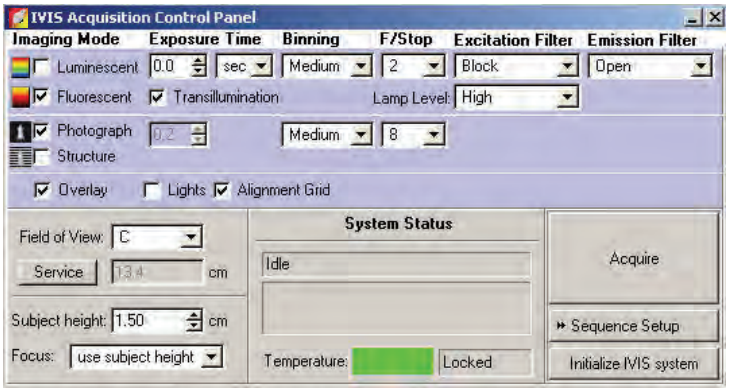
Imaging Mode	IVIS® Imaging System					See Page
	Lumina	100 Series	200 Series	Spectrum	3D Series	
Bioluminescent or fluorescent image.	✓	✓	✓	✓		18-19
Bioluminescent or fluorescent image.					✓	20-21
Bioluminescent image sequence. Fluorescent image sequence (reflectance mode)	✓	✓	✓	✓	✓	23-24
Fluorescent image sequence (transillumination mode)				✓		118

To acquire an image on the IVIS® Lumina, IVIS Spectrum, IVIS Imaging System 100 Series or 200 Series:

1. **For luminescent imaging:**
   a. Choose the Luminescent option.
   b. Confirm that the Excitation Filter is set to **Block** and the Emission Filter is set to **Open**.

   For fluorescent imaging:
   a. Choose the Fluorescent option.
   b. IVIS Spectrum only: Choose the Transillumination option if you want to use bottom illumination. For top illumination, leave this option unchecked.
   c. Choose the Filter Lock option and select the excitation filter from the drop-down list. The software automatically selects the appropriate emission filter.
   d. Confirm that the Fluorescent Lamp Level is set to **High**.

   Note: For more information on fluorescent imaging, see Appendix D, page 165.



2. **Set the binning:** Confirm the default binning level or select a new level for the luminescent or fluorescent image. For more details on binning, see page 13 and page 156.

3. **Set the exposure time:** Confirm the default exposure time or enter a new setting for the luminescent or fluorescent image. For more details on image exposure, see page 13 and page 156.

4. **Set the FOV:** To adjust the field of view (FOV), make a selection from the Field of View drop-down list. For more details on FOV, see pages 14, 156.
   Note: To view the subject(s) inside the chamber before image acquisition, take a photographic image (clear the luminescent or fluorescent option, choose the Photographic and Auto options, and click **Acquire**).

5. **Set the focus:** Do either of the following:
   - Select **use subject height** from the Focus drop-down list and use the ⇕ arrows or the keyboard arrows to specify a subject height (cm).

   or

   Select **Manual focus** from the Focus drop-down list. (For more details on manual focusing see Figure

6. **Set the photographic image settings:**
   a. Choose the Photographic option.
   b. Enter an exposure time or choose the Auto option.
   c. Confirm the binning and f/stop defaults or specify new settings for the photographic image.

7. If necessary, click ⇕ Image Setup in the control panel to operate in single image mode.
   Note: In single image mode, the ⇕ Sequence Setup button appears in the control panel. Click this button to set up sequence acquisition. (For more details on acquiring a sequence, see Figure 3.10, page 23.)

8. Click **Acquire**.
   During image acquisition, the **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.

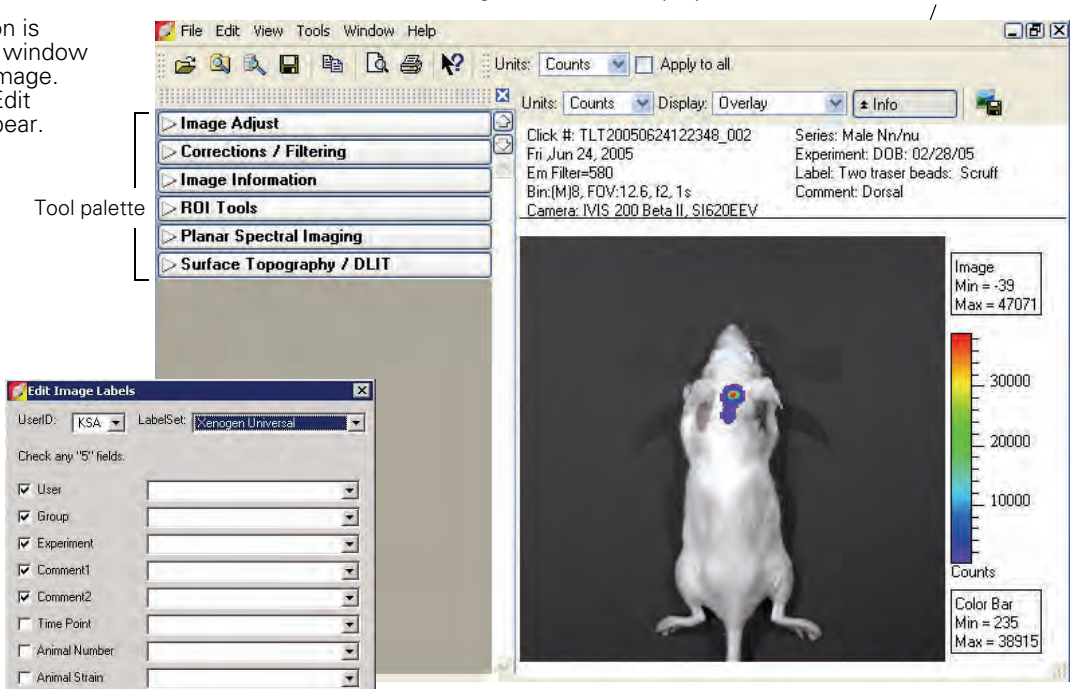
[continued in Figure 3.7]

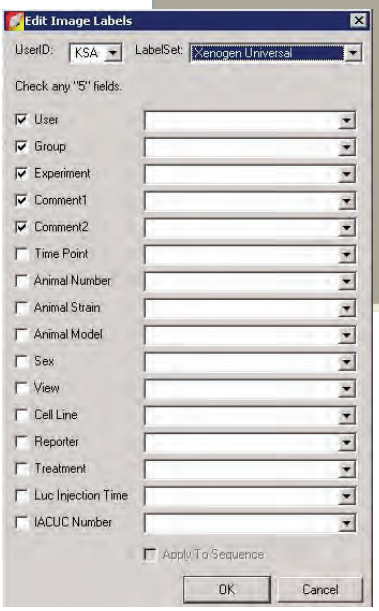
Figure 3.6 Acquiring an image on the IVIS Lumina, IVIS Spectrum, IVIS Imaging System 100 Series or 200 Series
Procedure continues in Figure 3.7.

To acquire an image on the IVIS® Lumina, IVIS Spectrum, IVIS Imaging System 100 Series or 200 Series (cont. from Figure 3.6):

Click **Info** in the image window to display the label set information.

After image acquisition is completed, the image window displays the overlay image. The tool palette and Edit Image Labels box appear.





Edit Image Labels box

9. Confirm that the signal of interest is above the noise level and below CCD saturation. (Check the image min and max at the top of the color bar).

   A signal of interest greater than 600 counts and less than ~60,000 counts is recommended. If the signal level is unacceptable, adjust the exposure time or binning level.

10. In the Edit Image Labels box that appears, enter the image information and click **OK**. If you do not want to enter label information, click **Cancel**.

Note: For details on how to save or export the image data, see Figure 3.17, page 29.

Figure 3.7 Acquiring an image on the IVIS Lumina, IVIS Spectrum, IVIS Imaging System 100 Series or 200 Series (continued from Figure 3.6)

To acquire an image on the IVIS® Imaging System 3D Series:

1. **For luminescent imaging:**
   a. Choose the Luminescent option.
   b. Confirm that the Excitation Filter is set to **Block** and the Emission Filter is set to **Open**.

   For fluorescent imaging:
   a. Choose the Fluorescent option.
   b. Choose the Filter Lock option and select the excitation filter from the drop-down list. The software automatically selects the appropriate emission filter.
   c. Confirm that the Fluorescent Lamp Level is set to **High**.

   Note: For more information on fluorescent imaging, see Appendix D, page 165.

2. **Set the binning:** Confirm the default binning level or select a new level for the luminescent or fluorescent image. For more details on binning, see page 13 and page 156.

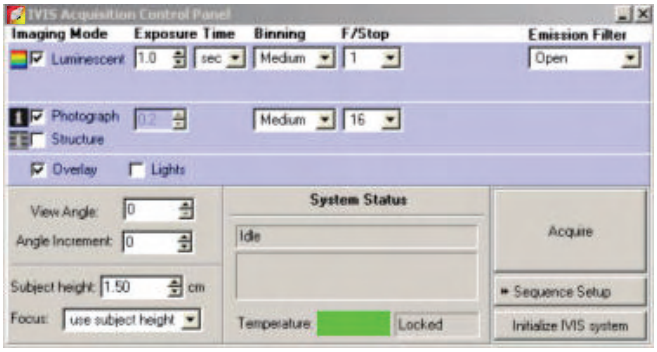
3. **Set the exposure time:** Confirm the default exposure time or enter a new setting for the luminescent or fluorescent image. For more details on image exposure, see page 13 and page 156.

4. **Set the focus:** Do either of the following:
   - Select **use subject height** from the Focus drop-down list and use the ▲▼ arrows or the keyboard arrows to specify a subject height (cm).

   or

   - Select **Manual focus** from the Focus drop-down list. (For more details on manual focusing see Figure 3.16, page 28.)

Note: To view the subject(s) inside the chamber before image acquisition, take a photographic image (clear the luminescent or fluorescent option, choose the Photographic and Auto options, and click **Acquire**).



5. **Set the photographic image settings:**
   a. Choose the Photographic option.
   b. Enter an exposure time or choose the Auto option.
   c. Confirm the binning and f/stop defaults or specify new settings for the photographic image.

6. If necessary, click Image Setup in the control panel to operate in single image mode.
   Note: In single image mode, the Sequence Setup button appears in the control panel. Click this button to set up sequence acquisition. (For more details on acquiring a sequence, see Figure 3.10, page 23.)

7. Click **Acquire**.
   During image acquisition, the **Acquire** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.

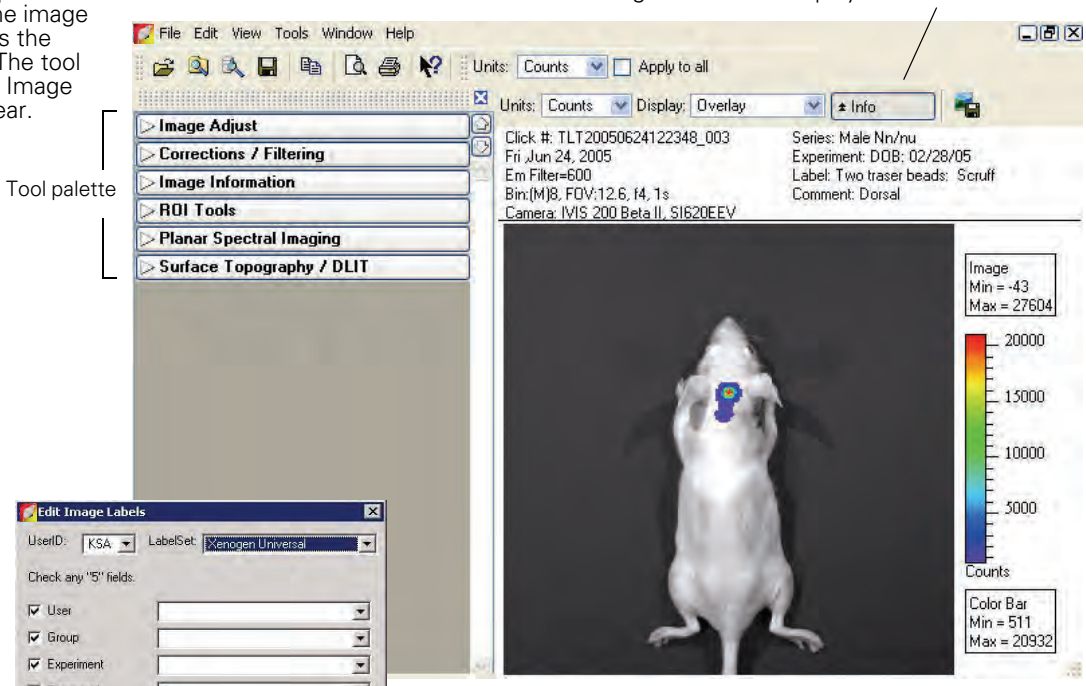
[continued in Figure 3.9]

Figure 3.8 Acquiring an image on the IVIS Imaging System 3D Series
Procedure continues in Figure 3.9.

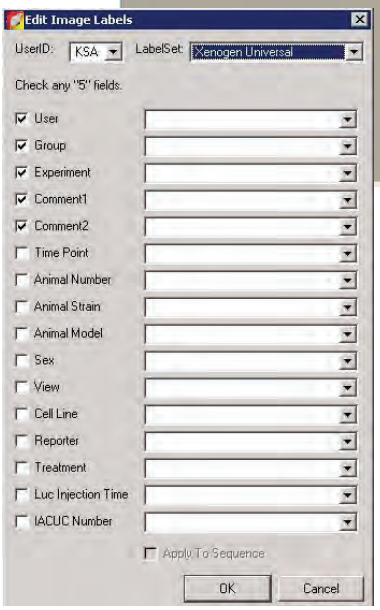
To acquire an image on the IVIS® Imaging System3D Series (cont. from Figure 3.8):

After image acquisition is completed, the image window displays the overlay image. The tool palette and Edit Image Labels box appear.

Click **Info** in the image window to display the label set information.



Tool palette



Edit Image Labels box

8. Confirm that the signal of interest is above the noise level and below CCD saturation. (Check the image min and max at the top of the color bar).

   A signal of interest greater than 600 counts and less than ~60,000 counts is recommended. If the signal level is unacceptable, adjust the exposure time or binning level.

9. In the Edit Image Labels box that appears, enter the image information and click **OK**. If you do not want to enter label information, click **Cancel**.

Note: For details on how to save or export the image data, see Figure 3.17, page 29.

Figure 3.9 Acquiring an image on the IVIS Imaging System 3D Series (continued from Figure 3.8)

Image Applications that Analyze an Image Sequence

Some types of image analysis require an image sequence (Table 3.7).

Figure 3.10 shows how to acquire an image sequence.

NOTE

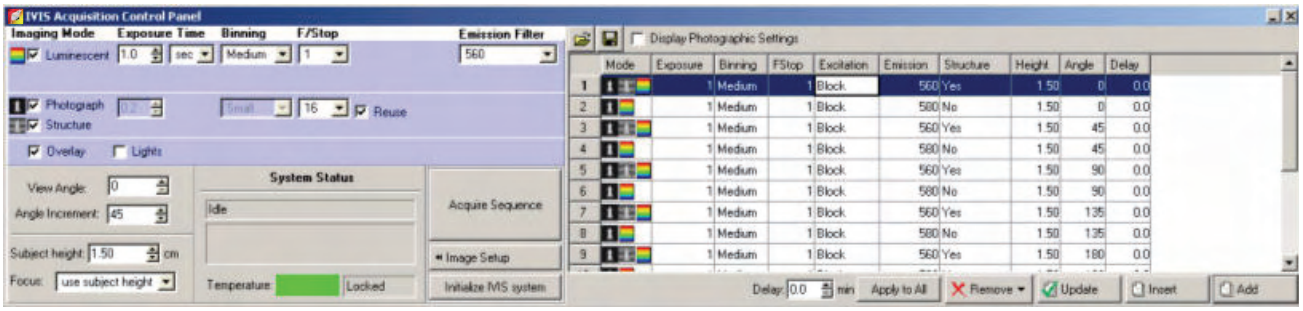
You can also create a sequence by grouping together images that were acquired during different sessions. For more details, see Chapter 4, page 38.

Table 3.7 Analyses performed on an image sequence

Type of Analysis	IVIS® Imaging System					For Image Sequence Requirements, See
	Lumina	100 Series	200 Series	Spectrum	3D Series	
Planar spectral imaging Computes the total flux and average depth of a source below the surface.		Optional	✓	✓	✓	Chapter 7, page 85
Display multiple fluorescent or luminescent reporters. Uses the Image Overlay function to display multiple luminescent or fluorescent images on one photographic image.	✓	✓	✓	✓	✓	Chapter 6, page 82
Subtract tissue autofluorescence using blue-shifted background filters. Uses the image math feature to subtract a background image from the primary image.	✓	✓	✓	✓	✓	Chapter 6, page 79
DLIT™ Analysis Reconstructs the surface topography of the subject and the brightness and 3D location of luminescent sources.			✓	✓	✓	Chapter 10, page 109
FLIT™ Analysis Reconstructs the surface topography of the subject and brightness and 3D location of fluorescent sources.				✓		Chapter 10, page 118
Spectral unmixing Removes tissue autofluorescence from a fluorescence image.				✓		Chapter 9, page 99

To acquire an image sequence on an IVIS® Imaging System:

1. Click Sequence Setup in the control panel to operate in sequence acquisition mode.
   - The sequence editor appears.



2. If necessary, click Remove and select **All** to clear the table.

3. In the control panel, specify the settings for the first bioluminescent or fluorescent image in the sequence and the photographic image:
   - For IVIS Imaging System100 Series, 200 Series, or Lumina: See Figure 3.6, page 18 for more details on image acquisition settings.
   - For IVIS System 3D Series: See Figure 3.8, page 20 for more details on image acquisition settings.

4. In the sequence editor, click Add.
   - The acquisition parameters are added to the table.

   Each row in the table specifies the acquisition parameters for one image in the sequence.

5. Repeat step 3 for each image in the sequence.

   Note: If you choose the Use Previous Photograph option in the control panel, the IVIS System acquires only one photographic image for the entire sequence. If this option is not chosen, the system acquires a photographic image for each image in the sequence.

6. To specify a time delay between each acquisition, enter a time in the Delay (min) box in the sequence editor.

7. If you want to save the sequence setup information (.xsq):

   a. In the sequence editor, click the **Save** button.

   b. In the dialog box that appears, select a directory for the file, enter a file name, and click **Save.**

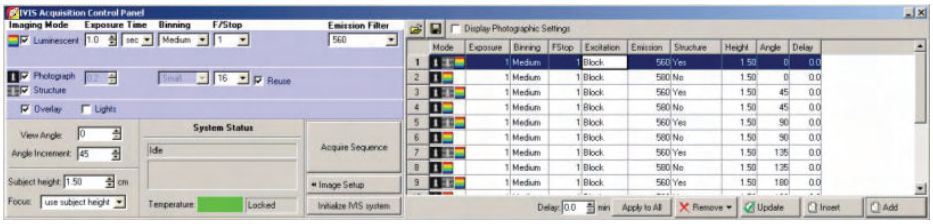
   Note: You can add or delete an image to the sequence, or edit a parameter value. For more details, see page 27 and 28.

[continued in Figure 3.11]

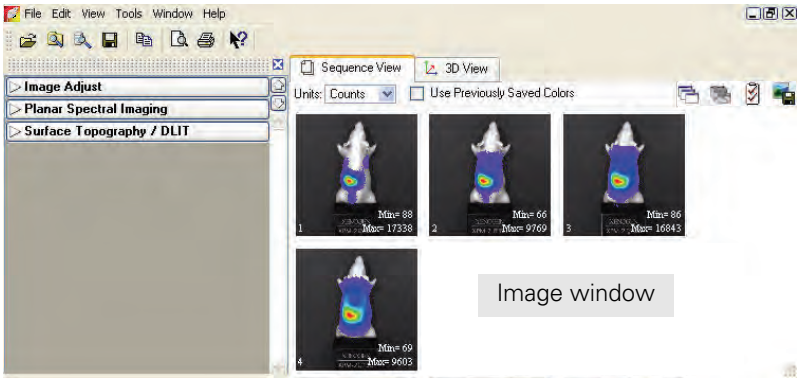
Figure 3.10 Acquiring an image sequence
Procedure continues in Figure 3.11.

To acquire an image sequence on an IVIS Imaging System (continued from Figure 3.10**):**

8. To acquire the images, click **Acquire Sequence** in the control panel.
   During image acquisition, the **Acquire Sequence** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.

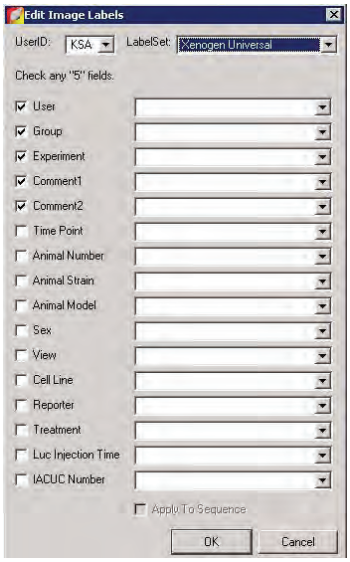


9. When all of the images are acquired and displayed in the image window, confirm that the signal of interest is above the noise level and below CCD saturation. (It is recommended that the signal of interest is greater then 600 counts and less than ~60,000 counts). If the signal level is unacceptable, adjust the exposure time or binning level.



10. In the Edit Image Labels box that appears, enter the image information and click **OK**. If you do not want to enter label information, click **Cancel**.

    Note: For details on how to save or export the image data, see Figure 3.17, page 29.

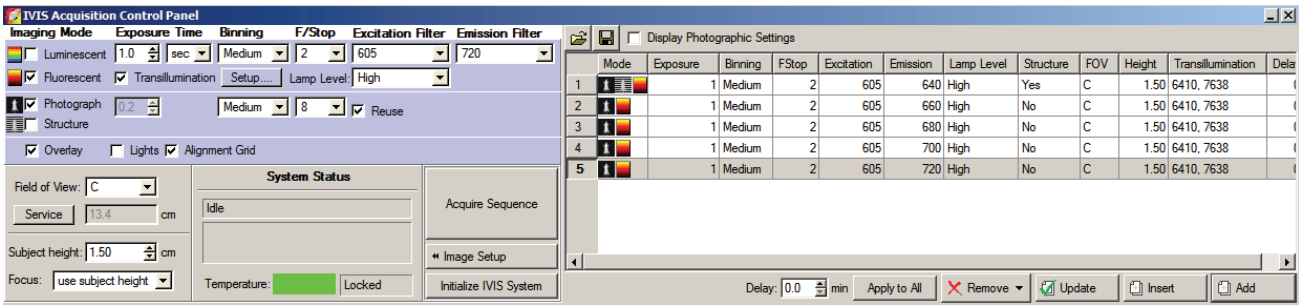


Edit Image Labels box

Figure 3.11 Acquiring an image sequence (continued from Figure 3.10**)**

To acquire a fluorescence image sequence in transillumination mode on the IVIS® Spectrum:

1. Click Sequence Setup in the control panel to operate in sequence acquisition mode.
   - The sequence editor appears.



2. If necessary, click Remove and select **All** to clear the table.

3. In the control panel, specify the settings for the fluorescence image (exposure time, binning, F/Stop, excitation filter, emission filter).

   For more details on image acquisition settings, see Figure 3.6, page 18.

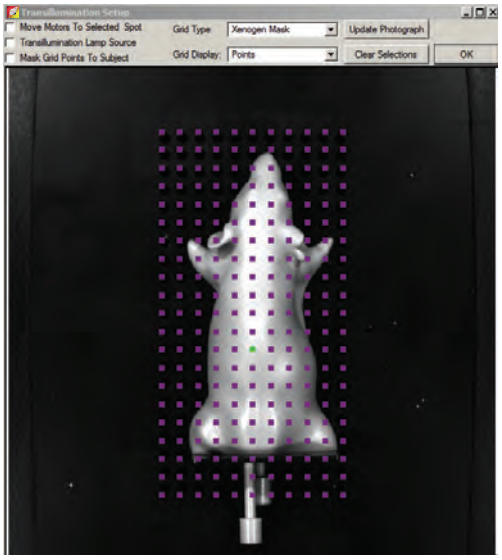
4. Specify the settings for the photographic image and choose the **Reuse** option.

5. In the control panel, select the Transillumination option and click **Setup**.
   - The Transillumination Setup window appears.

6. In the Transillumination Setup window, click the locations of interest (specifies the locations for transillumination and image acquisition).

7. In the sequence editor, click Add.
   - The acquisition parameters are added to the table.

   Each row in the table specifies one image in the sequence.



Transillumination setup window

8. If you want to save the sequence setup information (.xsq):
   a. In the sequence editor, click the **Save** button.
   b. In the dialog box that appears, select a directory for the file, enter a file name, and click **Save.**

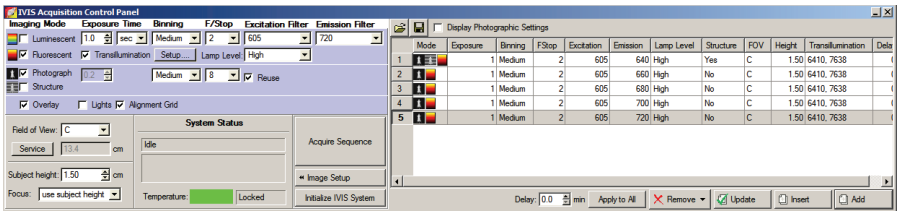
[continued in Figure 3.13]

Figure 3.12 Acquiring a transillumination fluorescence image sequence on the IVIS Spectrum
Procedure continues in Figure 3.13.

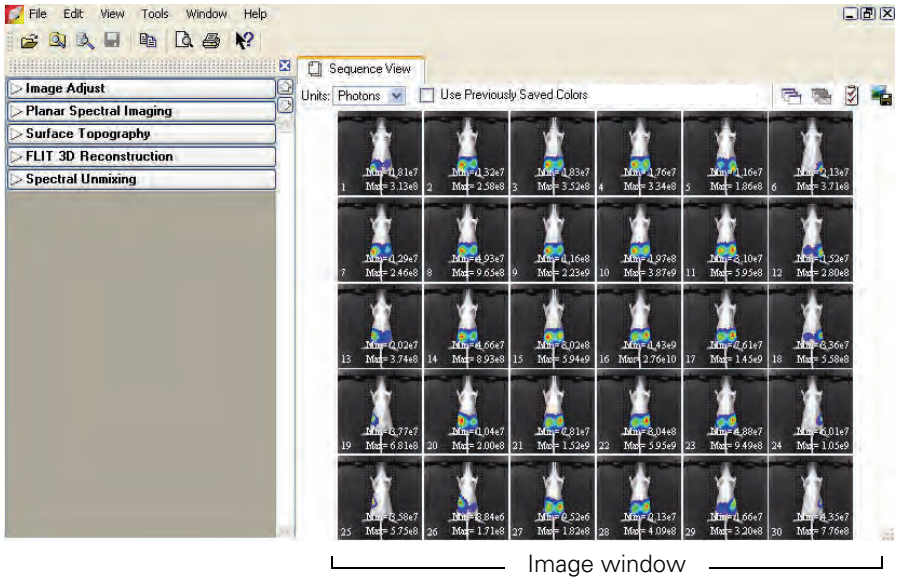
To acquire a fluorescence image sequence in transillumination mode on the IVIS® Spectrum (continued from Figure 3.12):

9. To acquire the images, click **Acquire Sequence** in the control panel.

   During image acquisition, the **Acquire Sequence** button becomes a **Stop** button. To cancel the acquisition, click **Stop**.



10. When all of the images are acquired and displayed in the image window, confirm that the signal of interest is above the noise level and below CCD saturation. (It is recommended that the signal of interest is greater then 600 counts and less than ~60,000 counts). If the signal level is unacceptable, adjust the exposure time or binning level.



11. In the Edit Image Labels box that appears, enter the image information and click **OK**. If you do not want to enter label information, click **Cancel**.

   Note: For details on how to save or export the image data, see Figure 3.17, page 29.

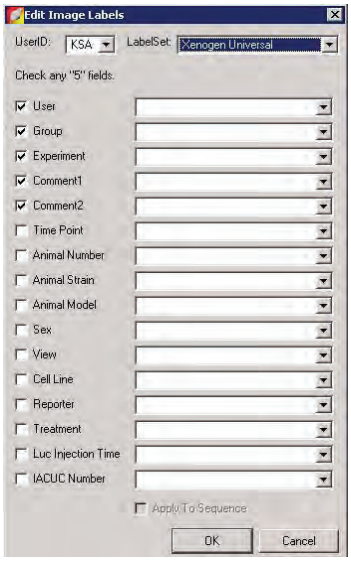


Figure 3.13 Acquiring a transillumination fluorescence image sequence on the IVIS Spectrum (continued from Figure 3.12)

Working In the Sequence Editor

In the sequence editor, you can add or delete an image to the sequence, or edit a parameter value (Figure 3.15).

NOTE

To specify an image acquisition sequence, it may be convenient to edit an existing sequence setup (.xsq) and save the setup to a new name.

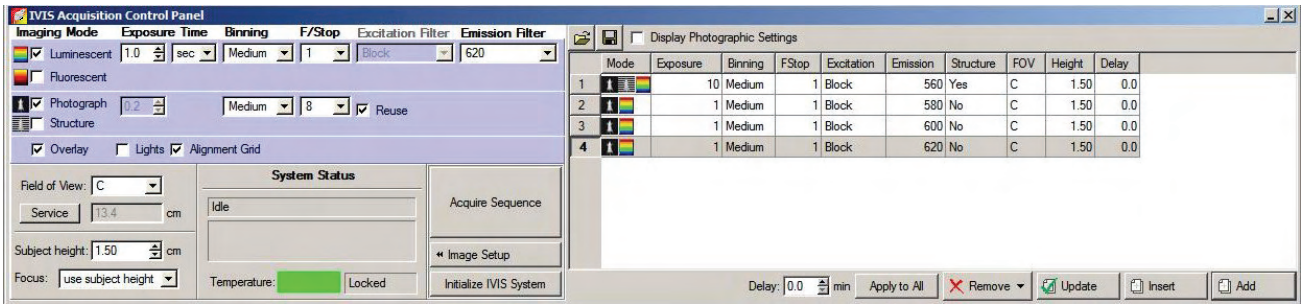
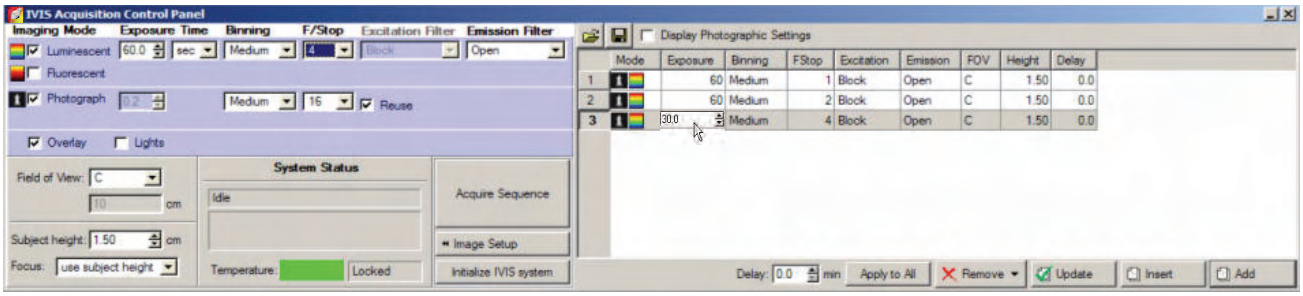


Figure 3.14 Control panel (left) and sequence editor (right)

Item in the Sequence Editor	Function
	Displays a dialog box that enables you to select and open a sequential setup (.xsq).
	Displays a dialog box that enables you to save a sequential setup (.xsq).
Display Photographic Settings	Choose this option to include the photograph exposure time, binning, and F/Stop in the sequence editor.
	Specifies a time delay between each image acquisition.
	Applies the selected cell value to all cells in the same column.
	Deletes the selected row from the Sequential Setup table.
	Updates the selected row in the Sequential Setup table with the acquisition parameters in the control panel.
	Inserts a row above the currently selected row using the information from the control panel.
	Adds a new row below the currently selected row using information from the control panel.



To edit a parameter value in the sequence editor:

1. Double-click the table cell that you want to edit.
2. Enter a new value in the cell or make a selection from the drop-down list.
3. Press **Enter** or click outside the cell.

Note: Press **Enter** on the keyboard to add a row to the end of the table using the information in the control panel.

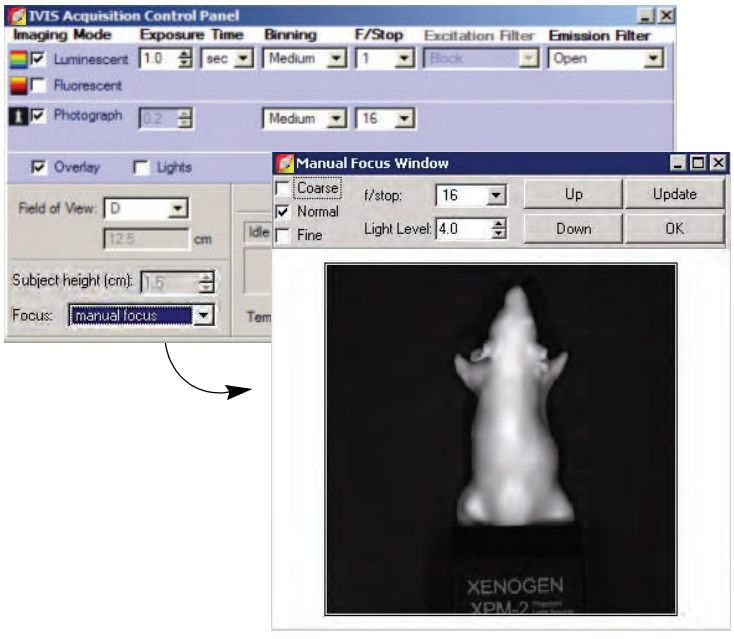
Figure 3.15 Editing a parameter value in the Sequential Setup table

3.6 Manual Focusing

The IVIS Imaging System automatically focuses the image based on subject height (see Focus, page 14). If you do not want to use the automatic focus feature, you can manually set the focus (Figure 3.16).

To manually set the focus:

1. In the control panel, choose **Manual Focus** in the Focus drop-down list.
   - The Manual Focus window appears.

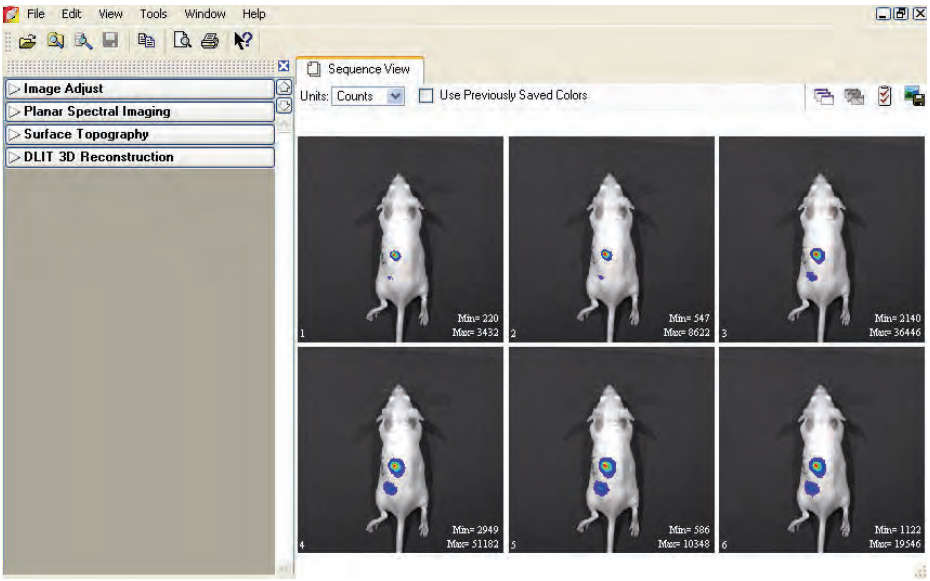


In the Manual Focus Window:

2. Select the size of the step increment that the stage moves, choose the **Coarse**, **Normal**, or **Fine** option.
3. Click **Up** or **Down** to move the stage and change the focus.
4. If necessary, select another f/stop setting from the drop-down list and adjust the light level using the ⇕ arrows.
5. Click **Update** to apply the settings.
   - The resulting focal plane (cm above the stage) is automatically entered in the Subject height box.
6. Click **OK** when the image is focused.

Figure 3.16 Manual focusing procedure

3.7 Saving or Exporting Image Data



To save the image data:

1. Click the **Save** button . Alternatively, select **File** →**Save** on the menu bar.
2. In the dialog box that appears, select a directory of interest and click **OK**.
   Note: The software automatically includes the user ID, and a date and time stamp with the data.

To export the image data to a graphic file (for example, .bmp):

1. Click the **Export Graphics** button .
2. In the dialog box that appears, select a directory, choose a file type, and enter a file name.
3. Click **Save**.

To export the image data to DICOM files (.dcm):

1. Select **File** →**Export** →**DICOM** on the menu bar.
2. In the dialog box that appears, select a destination (or create a new directory) for the data and click **OK**.
   - A directory containing the .dcm files and a SequenceInfo.txt is created at the specified location.

Figure 3.17 Saving or exporting image data

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4 Working With Images

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4.1 Opening Image Data

The Living Image® Browser provides a convenient way to browse image data, view information about the data, and open a single image or an image sequence (Figure 4.1).

You can also navigate to an image of interest and open it without the browser (see page 34).

Browsing Image Data

1. Click the **Browse** button . Alternatively, select **File →Browse** from the menu bar.
   - The Browse For Folder box appears.
2. In the Browse for Folder box, select the data of interest and click **OK**.
   Note: When you start the Living Image software and open the browser, the software automatically returns to the last folder visited.
3. The selected data are displayed in the Living Image browser along with the user ID and label information, and camera configuration information. The + sign indicates an image sequence.
4. Click the + sign to display the data in a sequence. To view data properties, right-click the item of interest and select **Properties** from the shortcut menu.

Note: For details on how to open an image or image sequence, see Figure

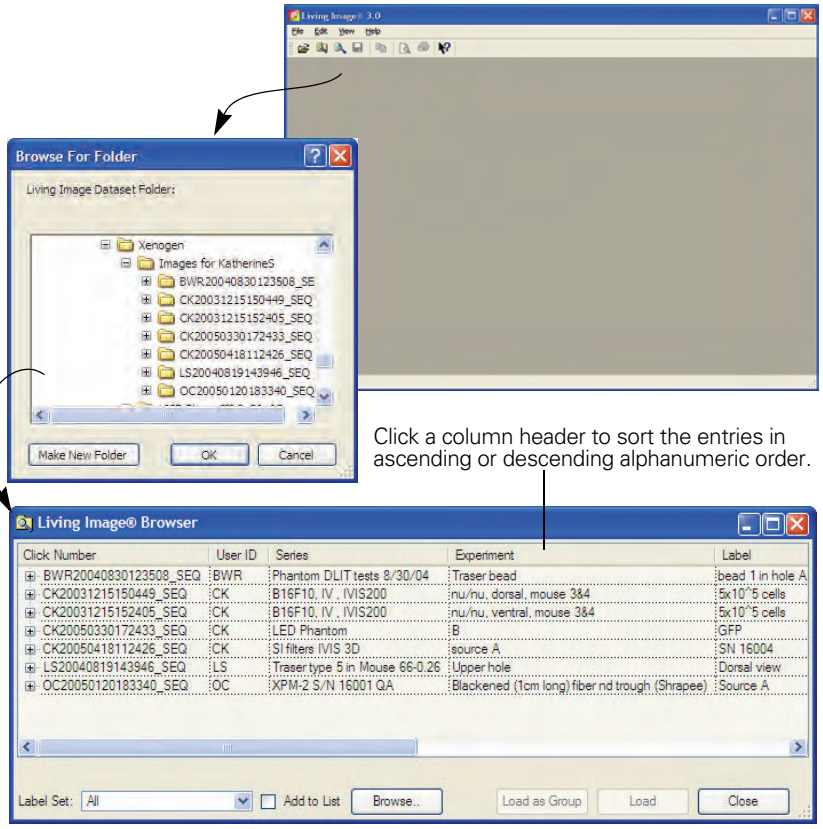


Figure 4.1 Browsing image data using the Living Image browser

Item in the Living Image Browser	Function
Label Set	Image information (column headers) displayed in the Living Image browser.
Add to List	When this option is chosen, data you select in the Browse for Folder box is added to the Living Image browser. If this option is not chosen, data selected in the Browse for Folder box overwrites the Living Image browser contents.
Load as Group	Enables you to select images acquired during different sessions and open them as a sequence. To select adjacent click numbers in the browser, press and hold the **Shift** key while you click the first and last file in the selection. To select non-adjacent click numbers in the browser: PC users press and hold the **Ctrl** key while you click the images of interest in the browser; Macintosh users press and hold the **Cmd** key (apple key) while you click the images of interest in the browser. Note: **Load as Group** is only available if single images not part of an image sequence are selected.
Browse	Opens the Browse For Folder box (Figure 4.1).
Load	Opens the selected image or image sequence.
Close	Closes the Living Image browser.

Opening Image Data With the Living Image Browser

Figure 4.2 shows how to open image data from the Living Image browser.

A sequence is a collection of images that are included in a single folder. A sequence may include images that were acquired during the same session and were intended to be grouped together. For example, a sequence might include a spectral filter scan, or images taken at different exposure time, or different angle views on the IVIS® Imaging System 3D Series.

Images acquired during different sessions can also be grouped together to form a sequence. For example, a time series could be constructed from images acquired on different days following an experimental treatment. (For more details, see *Creating an Image Sequence from Individual Images*, page 38.)

To open an image or image sequence:

1. In the Living Image browser, double-click the file or folder name. Alternatively, select the file or folder of interest and click **Load.**
   - At the start of a new session, the Select/Add User ID box appears.

   (For details on how to open the Living Image browser, see Figure 4.1.)

2. Enter your user ID or make a selection from the drop-down list, and click **OK**.
   - The image(s) and tool palette are displayed.

3. To open an image in a sequence, double-click the thumbnail.

   To open all images in a sequence, click the **Display All** button.

   To close all images, click the **Hide** button.

   For more details on working with a sequence in the image window, see page 36.

   For more information on the tool palette, see page 34.

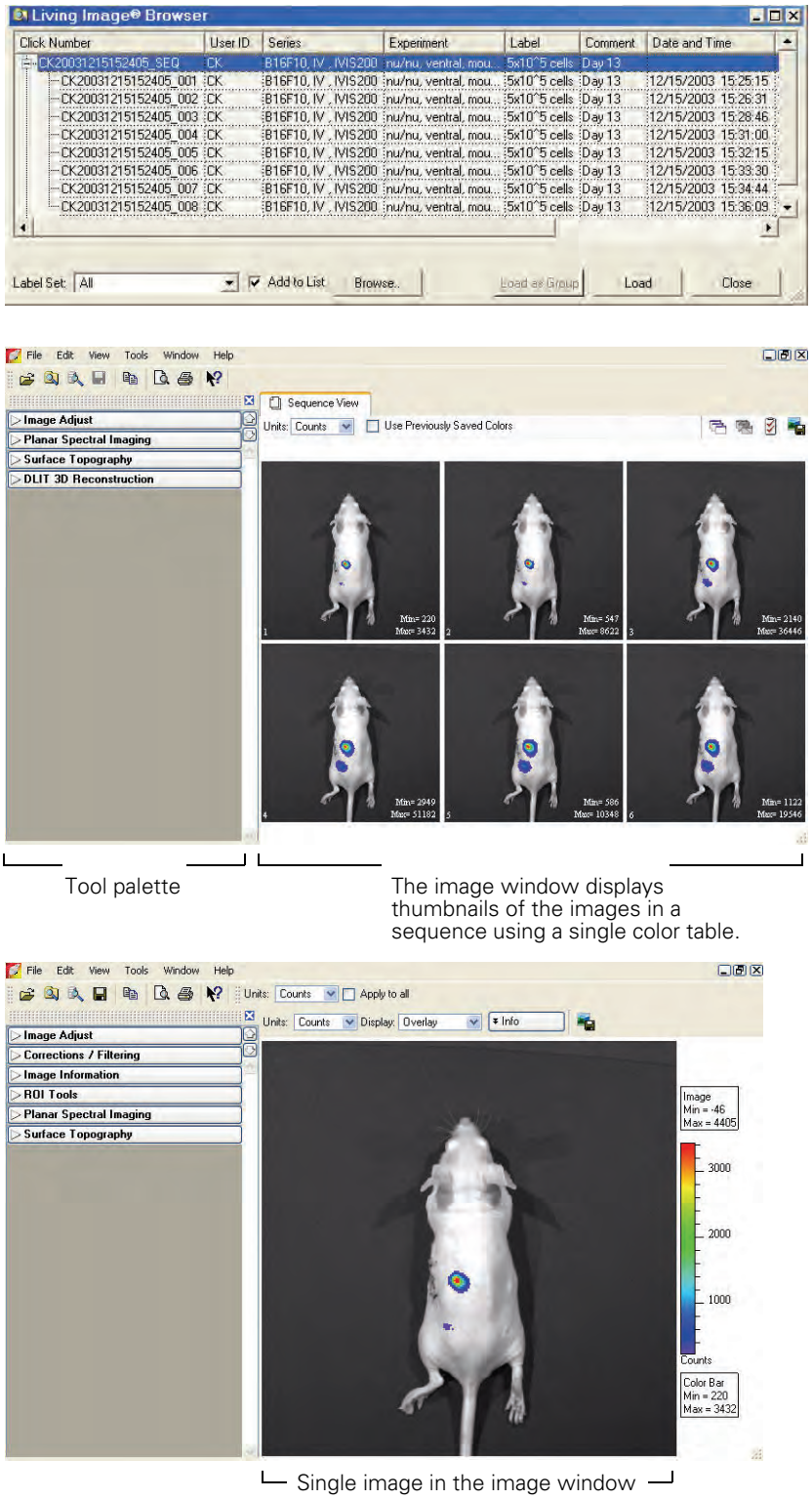
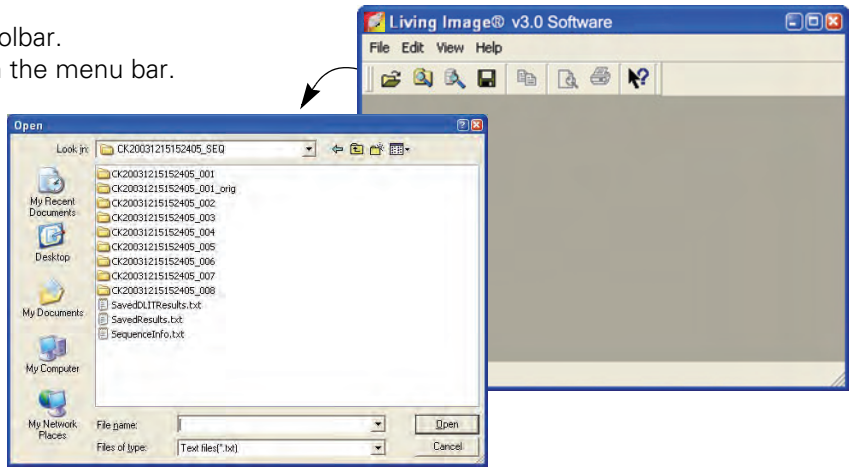


Figure 4.2 Opening an image sequence or an image
The tools available in the tool palette depend on the image data.

Opening Image Data Without the Living Image Browser

1. Click the **Open** button on the toolbar.
   Alternatively, select **File** →**Open** on the menu bar.
   - The Open box appears.



2. Double-click the data of interest.
   Alternatively, select the data and click **Open**.
   - At the start of a new session, the Select/Add User ID box appears.

3. Enter your user ID or make a selection from the drop-down list and click **OK**.
   - The image(s) and tool palette are displayed (Figure 4.2).

Figure 4.3 Opening image data without the Living Image browser

4.2 The Tool Palette

The tool palette contains information about the active image(s) and organizes the image analysis tools. The tools available in the tool palette depend on whether an image or an image sequence is active.

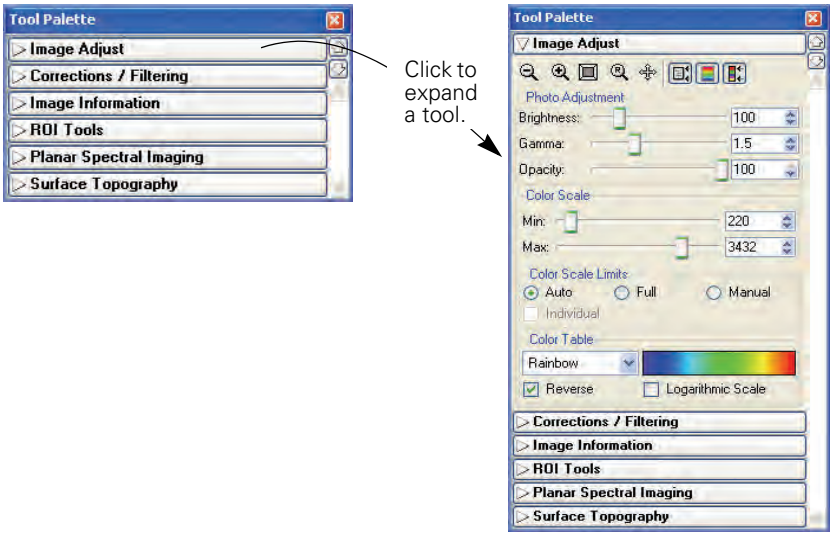


Figure 4.4 Tool palette

Undocking the Tool Palette

To change the position of the tool palette in the application window:

1. Position the pointer over the tool palette title bar so that it becomes a ✣.
2. Drag the tool palette to the new position in the main window and click.
   Note: To undock the tool palette, drag it a distance greater than its width.
3. To dock the tool palette to the left or right of the image window, drag the palette to the right or left of the main window and release.

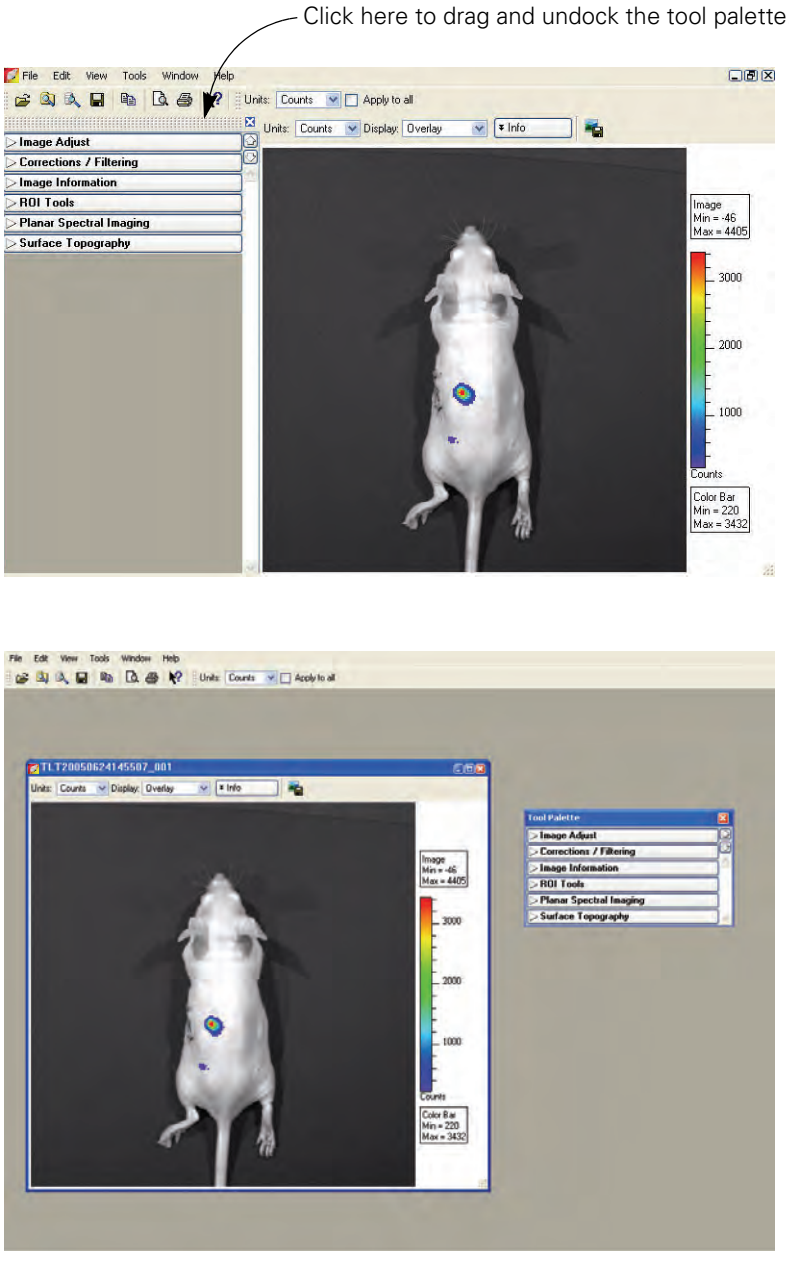


Figure 4.5 Tool palette docked (top) and undocked (bottom)
The tool palette can be docked at the left or right side of the main window.

4.3 Working With an Image Sequence

An image or image sequence is displayed in an image window. Multiple image windows can be open at the same time. The options available in the image window and tool palette depend on whether an image or an image sequence is displayed.

When you open an image sequence, the image window displays thumbnails of the images in the collection (Figure 4.6). A single color table is applied to the images. (For details on how to open an image sequence using the Living Image browser, see Figure 4.2, page 33.

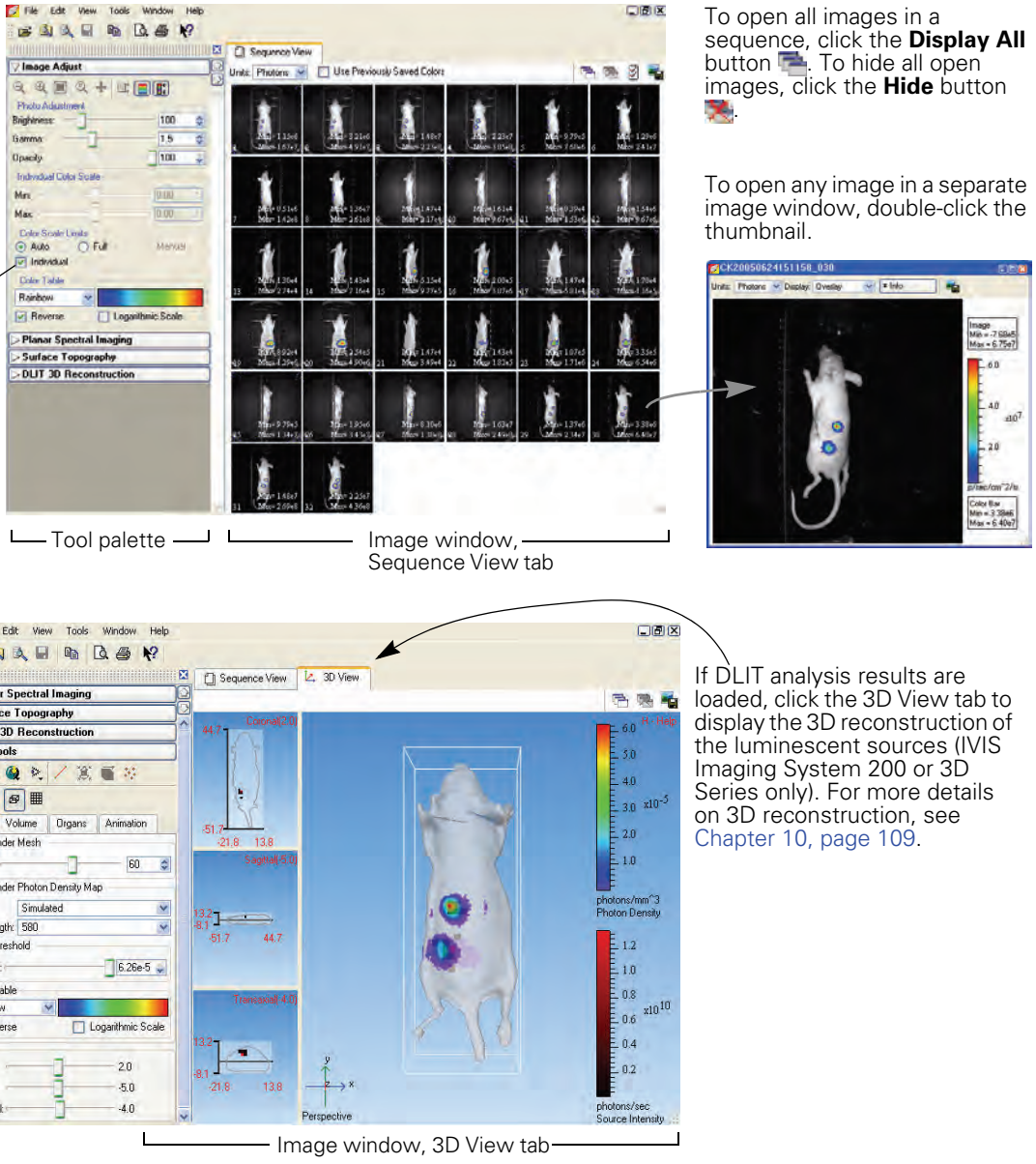
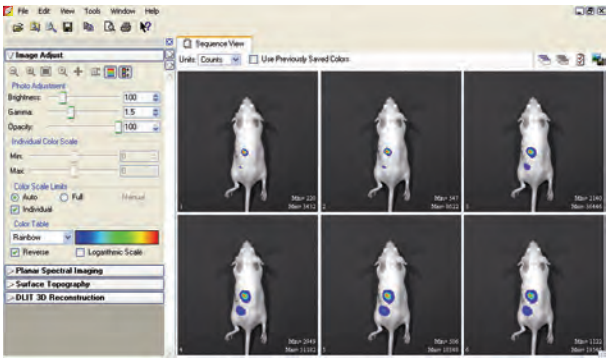

To open all images in a sequence, click the **Display All** button. To hide all open images, click the **Hide** button.
To open any image in a separate image window, double-click the thumbnail.
Choose **Individual** to apply a separate color scale to each image thumbnail in a sequence.
Tool palette
Image window, Sequence View tab
If DLIT analysis results are loaded, click the 3D View tab to display the 3D reconstruction of the luminescent sources (IVIS Imaging System 200 or 3D Series only). For more details on 3D reconstruction, see Chapter 10, page 109.
Image window, 3D View tab

Figure 4.6 Image sequence acquired on the IVIS Imaging System 3D Series (top) and DLIT 3D reconstruction of bioluminescent sources (bottom).

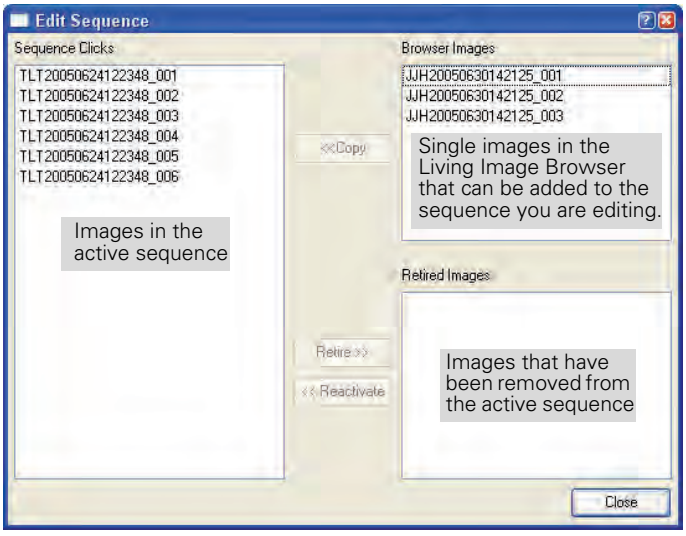
Item in the Image Window, Sequence View tab	Description
Units	Choose counts or photons for the image data from the drop-down list. For more details on counts and photons, see Appendix A, page 151.
Use Previously Saved Colors	Choose this option to display an image using the color table that was last applied to the image data.
	Click this button to open all images in a sequence.
	Click this button to close all open images in the active sequence.
	Click this button to open the Edit Sequence window that enables you to add or remove images from the active sequence. For more details on editing a sequence, see Figure 4.7, page 37.
	Click this button to export the active image in DICOM or other graphic file format (for example, .bmp, .jpg).

Editing an Image Sequence



To edit an image sequence:

1. Open the image sequence that you want to edit. (For details on how to open image data, see page 31).
2. If you want to add an image(s) to the sequence, brows for the image(s) in the Living Image browser.
   Note: Only individual images, not an image sequence, can be added to the sequence you are editing.
3. In the image window, click the **Edit** button .
   - The Edit Sequence window appears.



4. To remove an image from a sequence, select the click number in the Sequence Clicks windowpane, and click **Retire**.
   - The image is removed from the sequence and the file number is added to the Retired Clicks windowpane.

   Note: A retired image does not appear in the Living Image browser and cannot be added to another sequence.
5. To add an image to the sequence, select the click number in the Browser Clicks windowpane and click **Copy**.
   - The image is added to the active sequence and the click number is added to the Sequence Clicks windowpane.
6. When you are done editing the sequence, click **Close**.
   The sequence contents are updated and

Figure 4.7 Adding or removing images from a sequence

4.4 Creating an Image Sequence from Individual Images

To create a sequence from images acquired during different sessions:

1. In the Living Image Browser, browse for the images of interest. (For more details on browsing, see Figure 4.1, page 31.)

   Note: In the browser, an image that is a member of a sequence cannot be selected for grouping. For example, these images cannot be selected for grouping. Therefore, browse for individual images (which may or may not be part of a sequence), not image sequences.

2. In the browser, select the images that you want to group together.

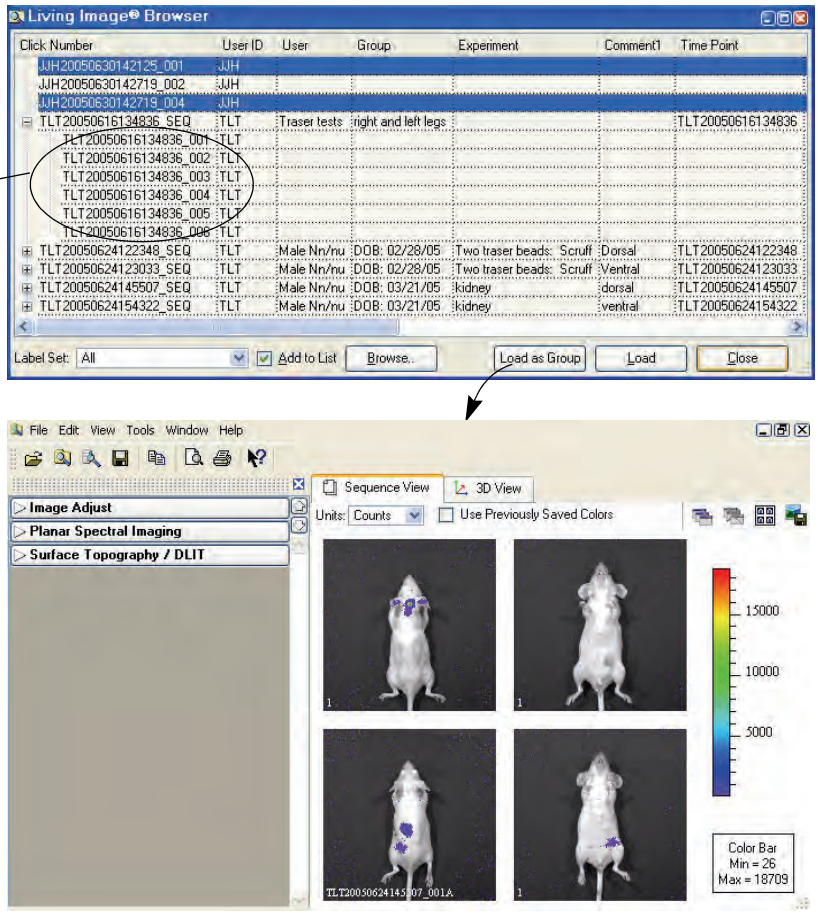
   - To select adjacent images in the browser, press and hold the **Shift** key while you click the first and last file in the selection.
   - To select non-adjacent images in the browser:

     PC users - Press and hold the **Ctrl** key while you click the images of interest in the browser.

     Macintosh users - Press and hold the **Cmd** key (apple key) while you click the images of interest in the browser.

3. Click **Load as Group**.

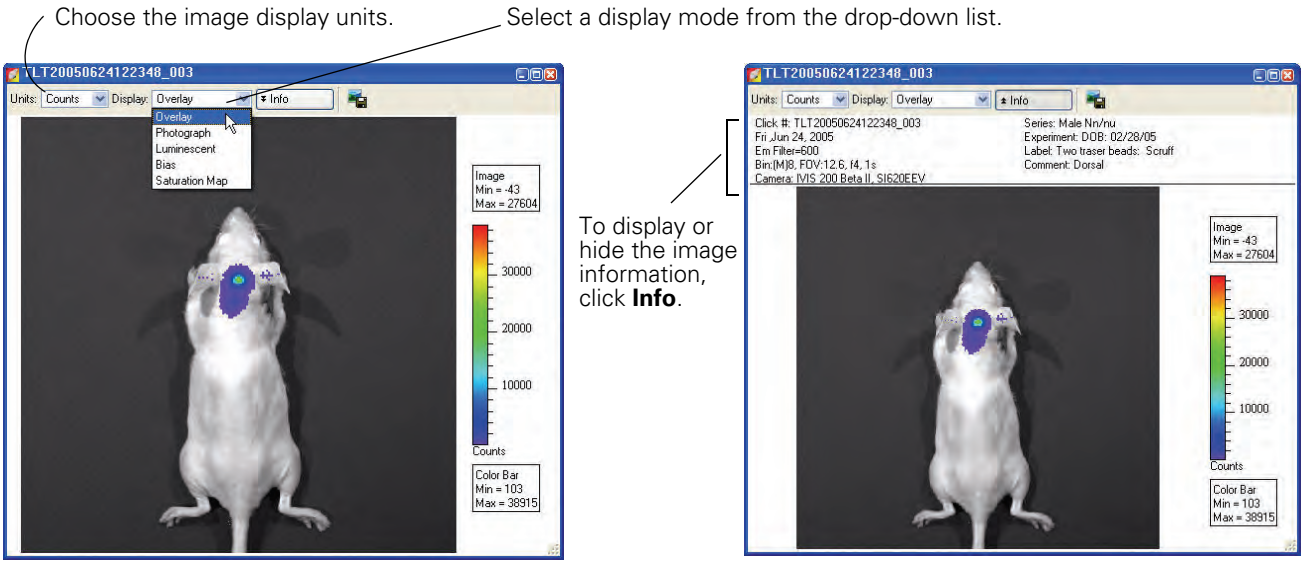
   - The image thumbnails are displayed together in an image window.



Note: For details on how to save or export the image data, see Chapter 3, page 29.

Figure 4.8 Creating an image sequence from images acquired during different sessions

4.5 Working With a Single Image



Note: For details on how to browse data and open images, see Figure 4.1, page 31 and Figure 4.2, page 33.

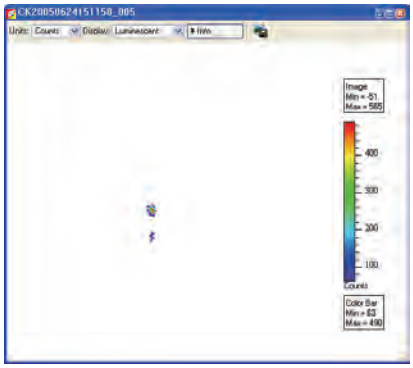
Figure 4.9 Image window, overlay display mode

Item in the Image Window	Description
Units	Choose counts or photons from the drop-down list for the image data. For more details on counts and photons, see Appendix A, page 151.
Display	To choose an image display mode in the image window, make a selection from the Display drop-down list (Figure 4.9). See Figure 4.10 for examples of the display modes.
Overlay	A pseudocolor image of luminescent or fluorescent image data displayed over a grayscale photographic image.
Photograph	A grayscale image that is captured when the IVIS® Imaging System illumination lights are activated.
Luminescent	A pseudocolor image of the luminescent or fluorescent data captured during an exposure when the IVIS Imaging System illumination lights are off.
Fluorescent	A pseudocolor image of the fluorescent data captured during an exposure when the IVIS Imaging System illumination lights are off.
Background	The CCD camera background acquired with the camera shutter closed. (See Appendix C, page 159.)
Bias	An electronic offset that exists on every pixel. This means that the zero photon level in the readout is not actually zero, but is typically a few hundred counts per pixel. The read bias offset is reproducible within errors defined by the read noise, another quantity that must be determined for quantitative image analysis.
Saturation Map	Displays image regions that saturated the CCD digitizer (>65535 counts) in red. ROI measurements should not be made on saturated regions. ROI measurements made on image regions that do not contain saturated pixels are accurate (unless the image is badly saturated).
Structure	A structured light image of parallel laser lines scanned across the subject. (Available in the IVIS® Imaging System 200 Series and 3D Series.) The surface topography of the subject is determined from the structured light image.
Reference	A structured light image of a white plate that is acquired and stored on disk prior to instrument installation.
3D View	A three-dimensional rendering of the subject. For more details see Appendix F, page 189.

Item in the Image Window	Description
Export	Opens the Export Active View As Image box so that the active image data can be exported (bmp, jpg, png, tiff, or postscript format).
Info	Click to display or hide information about the image in the image window (Figure 4.14).

Overlay

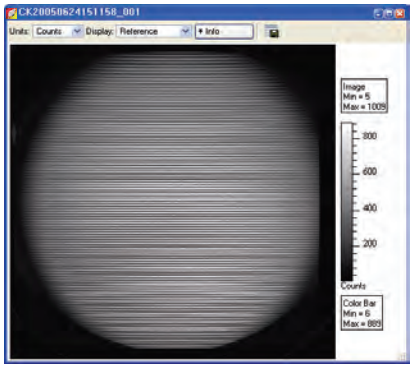
Photograph



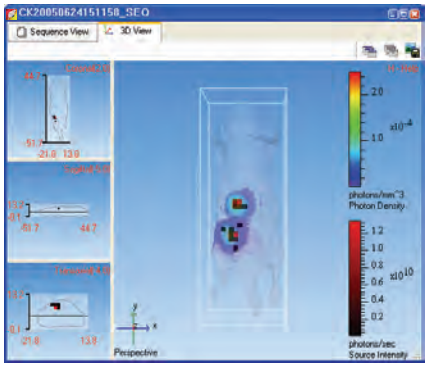
Luminescent

Saturation Map

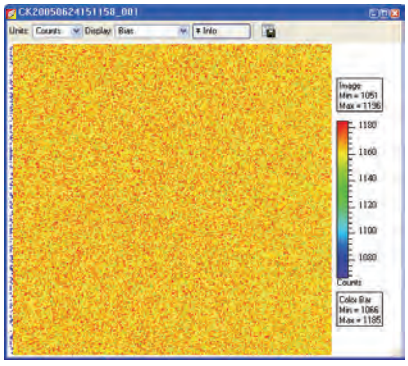
Structure



Reference



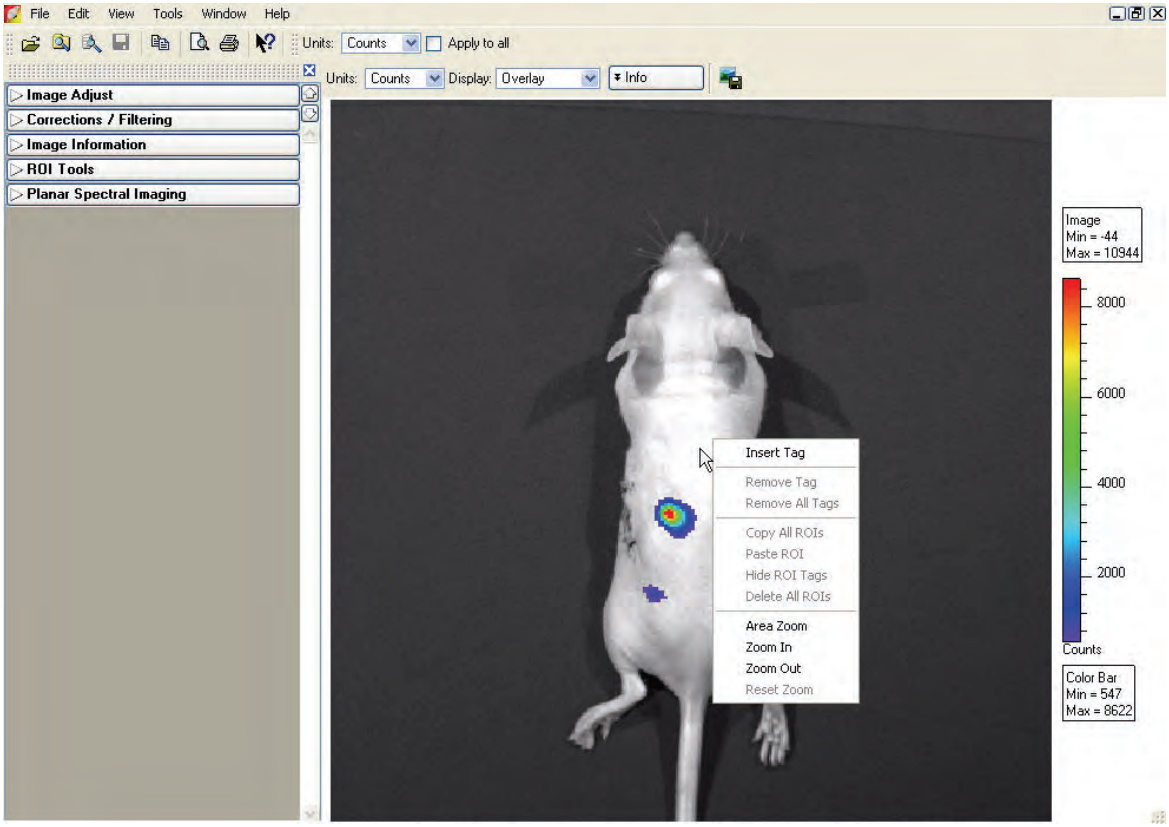
3D View



Bias

Figure 4.10 Display modes for a single image
The software coregisters the luminescent and photographic image to generate the overlay image.

Magnifying or Panning in the Image Window



To incrementally zoom in or out on an image:

- Click the or button. Alternatively, right-click the image and select **Zoom In** or **Zoom Out** on the shortcut menu.

To magnify a selected area in an image:

1. Click the button. Alternatively, right-click the image and select **Area Zoom** on the shortcut menu.
   - The pointer changes to a +.
2. Draw a rectangle around the area that you want to magnify.
   - The selected area is magnified when you release the mouse button.

To reset the magnification (remove magnification):

- Click the button. Alternatively, right-click the image and select **Reset Zoom** on the shortcut menu.

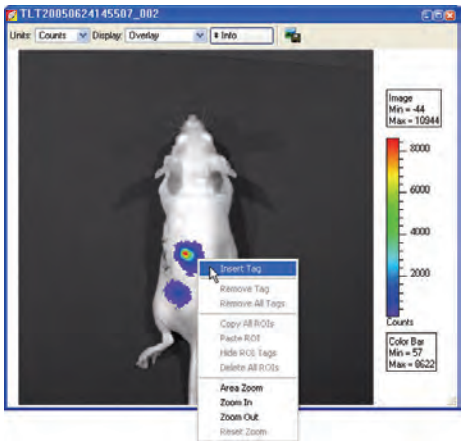
To pan the image window:

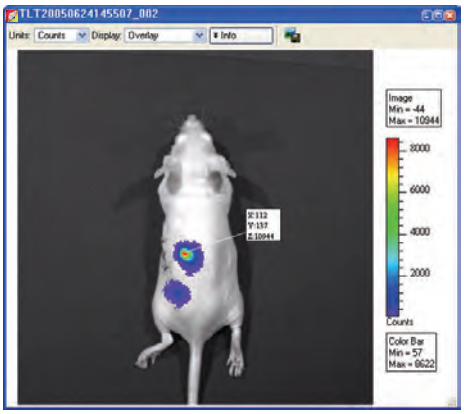
1. Click the button.
   - The pointer becomes a .
2. To move the image in the image window, click and hold the pointer while you move the mouse.
   Note: This feature helps you view different areas of a magnified image. If the image has not been magnified, you cannot pan the image.

Figure 4.11 How to magnify an image or pan the image window

Tagging an Image

You can apply a tag to a user-specified location in an image (Figure 4.12). The tag displays the x,y pixel coordinates of the location, and the pixel intensity (z, counts or photons).





To tag a location:

1. Right-click the location of interest in the image.
2. Select **Insert Tag** on the short cut menu.

To remove a tag:

1. Position the pointer over the tag.
2. Right-click the image and select **Remove Tag** on the shortcut menu.
3. To remove all tags, right-click the image and select **Remove All Tags** on the shortcut menu.

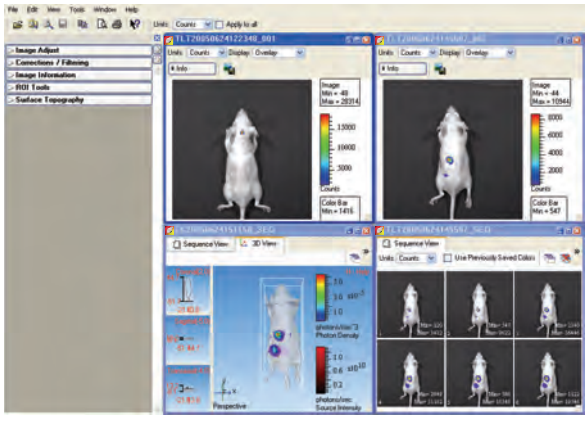
To move the tag:

1. Position the pointer over the label.
2. When the hand tool appears , use a click-and-drag operation to move the tag, then click the mouse to set the tag location.
   - A line between the pixel and the tag identifies the location associated with the tag.

Figure 4.12 Applying a tag to an image

Organizing Images

When multiple image windows are open, you can organize them in a cascade or tile arrangement (Figure 4.13).



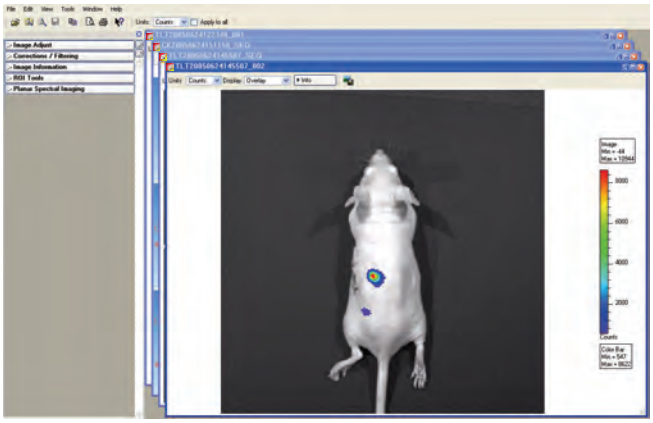


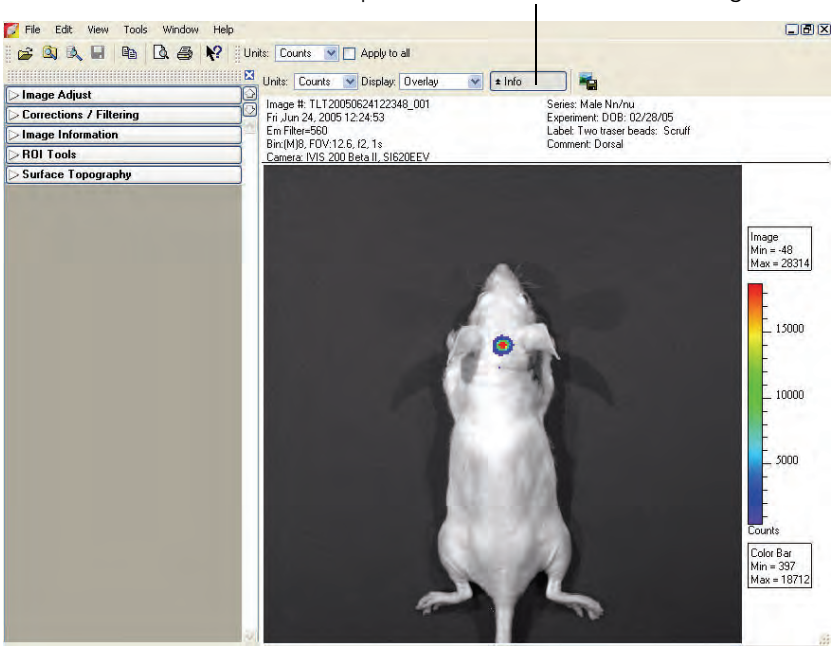
Figure 4.13 Image windows: tiled (left) or cascade (right)

4.6 Viewing Image Information

Information about an image that is captured at acquisition includes all of the text information that is saved with every image (for example, camera parameters and user labels).

To view information about an image:

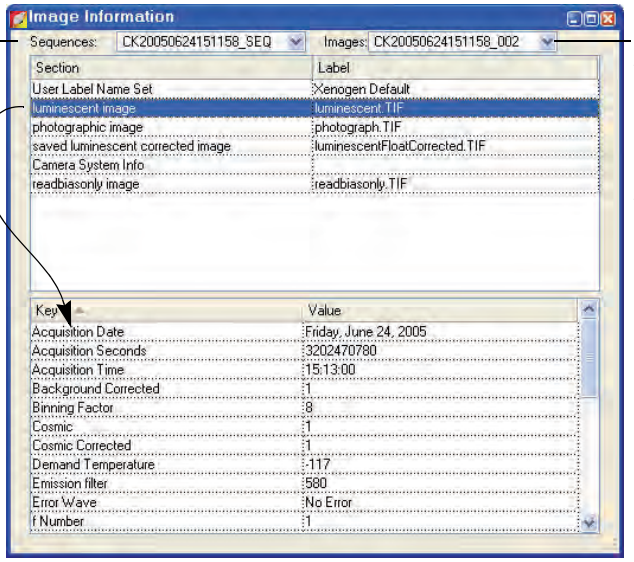
1. Open the image or image sequence of interest. (For details on how to open data, see Figure 4.2).
2. Select **View →Image Information** on the menu bar.
   - The Image Information window appears.



List of open image sequences. Choose **Individual Images** from the list to show the open single images in the Images drop-down list.

1. To choose an image, make a selection from the Sequences drop-down list and the Images drop-down list.
2. To view information of interest, click a category in the upper box.
   - The information is displayed in the lower box.

   For example, if you select luminescent image, the luminescent image acquisition parameters are displayed.



List of images in the selected sequence or open single images if Individual Images is selected from the Sequences drop-down list.

Figure 4.14 Viewing the image information

Editing Image Label Information

You can edit user label information after image acquisition.

1. Open the image of interest.
2. Select **Edit →Image Labels** on the menu bar.
   - The Edit Image Labels box appears.
3. In the Edit Image Labels box, edit the information of interest. You can also select a new label set to apply to the image or

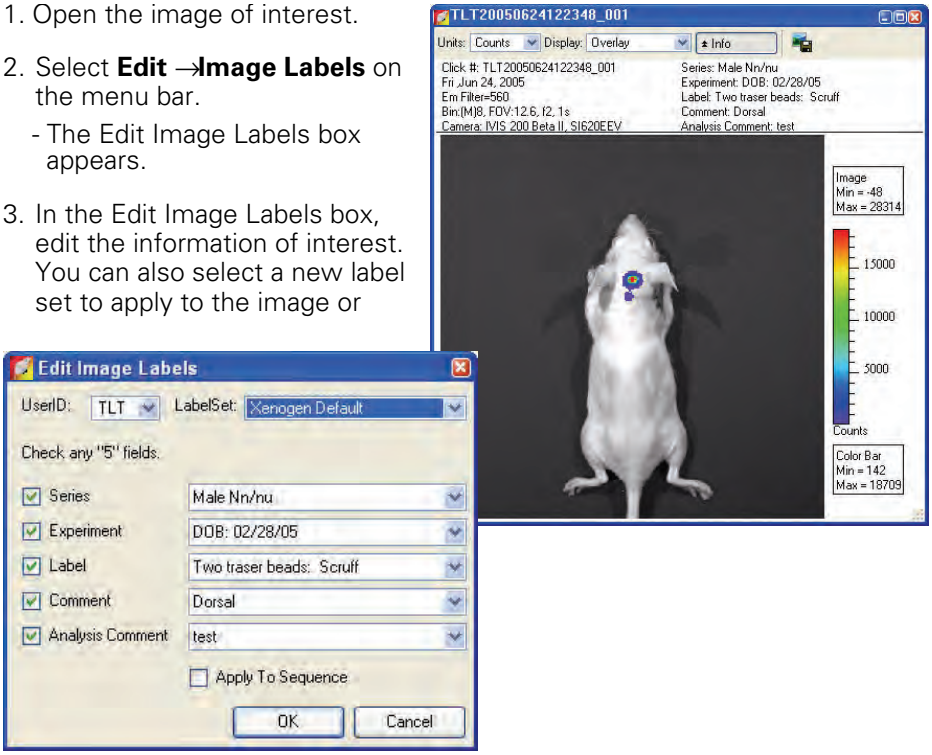


Figure 4.15 Editing image label information

4.7 Adjusting Image Appearance

You can adjust the appearance of an image using the Image Adjust tools (Figure 4.16).

NOTE

Not all tools are available for all image display modes.

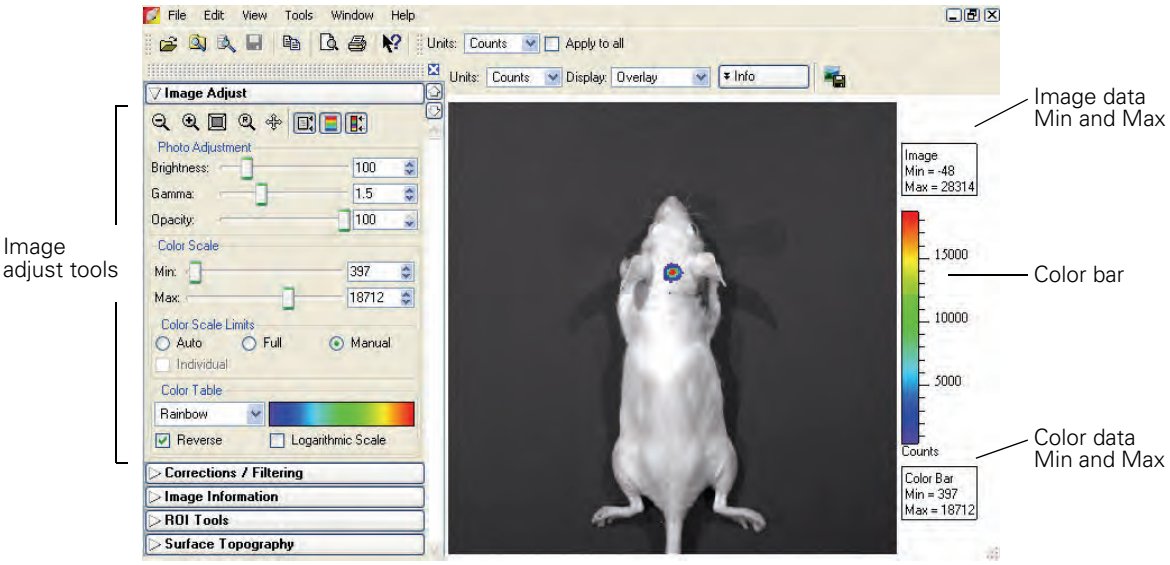


Figure 4.16 Tool palette, image adjust tools

Image Adjust Tool	Description
	Click this button to incrementally zoom out on the image (reduces the image dimensions in the image window). Note: The zoom tools are also available in the shortcut menu when you right-click the image (**Ctrl**-click for Macintosh users).
	Click this button to incrementally zoom in on the image (incrementally magnifies the image in the image window).
	Click this button to magnify the area inside a rectangle that you draw using a click-and-drag operation. (Sets the dimensions of the magnified area equal to image window dimensions.)
	Click this button to return the image to the default display magnification.
	Click this button to move a magnified image (*pan*) in the image window. For more details, see Figure 4.11.
	Click this button to hide or display the image min/max information in the image window
	Click this button to hide or display the color scale in the image window
	Click this button to hide or display the color scale min/max information in the image window

Image Adjust Tool	Description
Photo Adjustment	
Brightness	Click and move the slider left or right to adjust the brightness of an image displayed in overlay or photograph mode. Alternatively, enter a brightness value.
Gamma	Click and move the slider left or right to adjust the *gamma* of a image displayed in overlay mode. Alternatively, enter a gamma value. (Gamma is related to image contrast.)
Opacity	Click and move the slider left or right to adjust the opacity of the pseudocolor luminescent data of an image displayed in overlay mode. Alternatively, enter an opacity value.
Color Scale	
Min	The minimum pixel intensity associated with the color bar for an image. Pixels less than the minimum value are not displayed.
Max	The maximum pixel intensity associated with the color bar for an image. Pixels greater than the maximum value are not displayed.
Limits	
Auto	When this option is chosen, the software sets the Min and Max values to optimize image display and suppress background noise. The Min and Max settings can be manually adjusted to further optimize the image display for your needs.
Full	Choose this option to set the Max and Min values to the maximum and minimum data values in the image.
Manual	Choose this option to enter Max and Min values for the image display.
Individual	Applies a separate color table to each image in a sequence. Note: This option is only available when an image sequence is active.
Color Table	
	Click the drop-down arrow to select a color table for the image data. (For more details on color tables, see *Pseudocolor Images*, page 149.)
Reverse	Choose this option to reverse the min and max colors of the selected color table.
Logarithmic Scale	Choose this option to apply a log scale to the relationship between numerical data and the color range in the color table. A log scale increases the range of meaningful numerical data that can be displayed.

4.8 Correcting or Filtering Image Data

The Corrections/Filtering tools enable you to subtract background or apply corrections to the image data. (For more details on sources of background, see Appendix C, page 159.) You can also apply smoothing and soft binning to the image data. (For more information on binning and smoothing, see Appendix B, page 155.)

- To view the tools, click **Corrections/Filtering** in the tool palette (Figure 4.17).

Read Bias Subtraction and Flat Field Correction are mandatory defaults in photons mode. In counts mode, these corrections can be cleared.

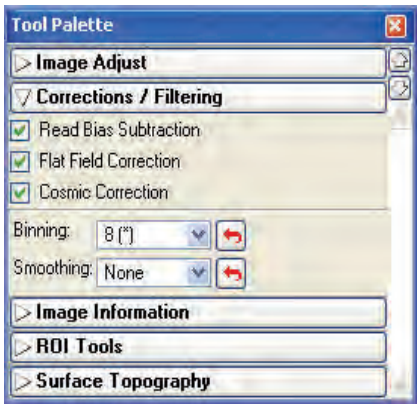
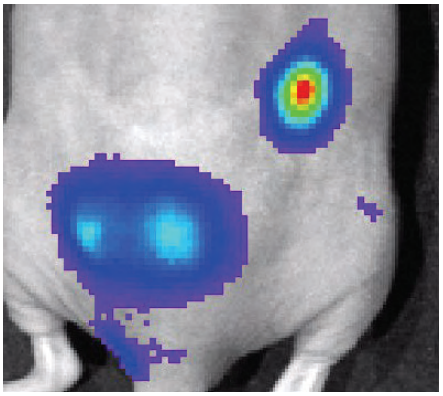
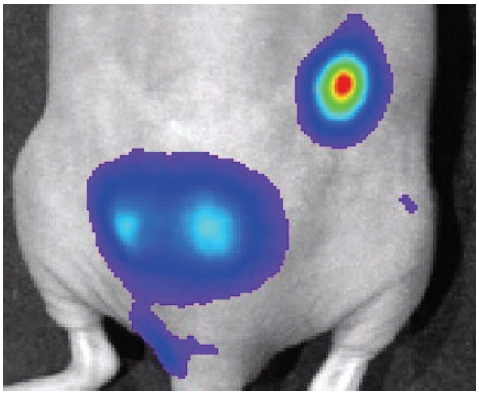


Figure 4.17 Tool palette, Corrections/Filtering tools

Corrections/Filtering Tool	Description
Read Bias Subtraction	Select this check box to subtract dark background from the image data. For more details on background, see Appendix C, page 159. Note: In photons mode, dark background subtraction is a mandatory default. In counts mode, the check box can be cleared.
Flat Field Correction	Select this check box to apply a lens correction factor to the image data. For more details on flat field correction, see Appendix A, page 153. Note: In photons mode, flat field correction is a mandatory default. In counts mode, the check box can be cleared.
Cosmic Correction	Select this check box to correct image data for cosmic rays or other ionizing radiation that interact with the CCD. For more details on cosmic correction, see Appendix A, page 154.
Binning	Specifies the number of pixels in the image data that are grouped together to form a larger pixel (called *soft* binning). Binning changes the pixel size in the image (Figure 4.18). For more details on binning, see Appendix B, page 156.
Smoothing	Computes the average signal of the specified number of pixels and replaces the original signal with the average signal (Figure 4.18). Smoothing removes signal noise without changing pixel size. **Note:** This type of smoothing is defined differently from the smoothing performed in the Living Image® 2.5 software.
	Click this button to return the binning or smoothing to the previous setting and update the image.



Binning at acquisition = 8, no smoothing



Binning = 2, smoothing = 5x5

Figure 4.18 Example of binning and smoothing image data

4.9 Image Information Tools

You can view information about the active image using the Image Information tools. The tools enable you to make measurements in an image and view pixel data in different formats.

Image Information	Description	See
x,y coordinates and associated intensity	The x,y pixel coordinates of the mouse pointer location in the image and the intensity (counts or photons) at that location.	Figure 4.20, page 50
Histogram	Histogram of pixel intensities in an image.	Figure 4.21, page 51
Line profile	Plots a line graph of intensity data at each pixel along a user-specified horizontal or vertical line in the image	Figure 4.22, page 52

- To view the tools, click **Image Information** in the tool palette (Figure 4.19).

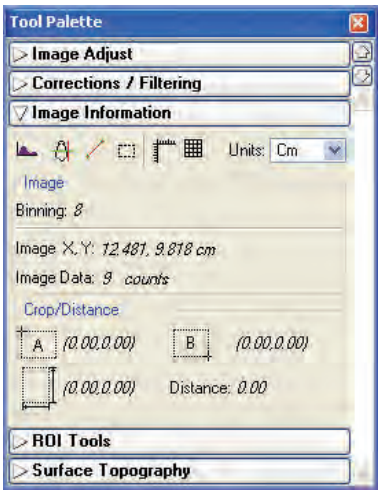


Figure 4.19 Tool palette, Image Information tools

Image Information Tool	Description	See
	Click this button to display a histogram of pixel intensity.	Figure 4.21, page 51
	Click this button to display a line profile.	Figure 4.22, page 52
	Click this button to display the distance measurement cursor in the image window.	Figure 4.23, page 53
	Click this button to draw and measure a rectangle on an image.	Figure 4.24, page 54
	Click this button to display/hide a scale on the x and y-axis of the image window.	Figure 4.20, page 50
	Click this button to display/hide a grid the image window.	Figure 4.20, page 50

Image Information Tool	Description	See
	Choose the units (cm or pixels) for distance measurements in the image window.	
Image		
Binning	The binning applied to the image. **Note:** If soft binning is applied to the image data, and the binning level is changed from 8 to 16, the new binning is indicated as 8x2.	
Image X,Y	The x,y pixel coordinates of the mouse pointer location in the image.	Figure 4.20, page 50
Image Data	The intensity (counts or photons) at the pixel location of the mouse pointer.	Figure 4.20, page 50
Crop/Distance		
	The x,y pixel coordinates at the upper left corner of the crop tool. OR The x,y pixel coordinates at the "A" end of the distance measurement cursor.	Figure 4.24, page 54 Figure 4.23, page 53
	The x,y pixel coordinates at the lower right corner of the crop tool. OR The x,y pixel coordinates at the "B" end of the distance measurement cursor. For details see.	Figure 4.24, page 54 Figure 4.23, page 53
	The width and height of the image crop tool. OR Δx, Δy from the A to B end of the distance measurement tool.	Figure 4.24, page 54 Figure 4.23, page 53
Distance	The length of the diagonal from corner A to corner B in the image crop tool. OR The length of the distance measurement cursor.	Figure 4.24, page 54 Figure 4.23, page 53

Viewing X,Y Coordinates & Intensity Data

To view x,y coordinates information & associated intensity:

1. In the Image Informations tools, choose Cm or Pixels from the Units drop-down list.
2. Put the mouse pointer over the location of interest in the image.
   - The x,y coordinates and intensity data are displayed in the tool palette.

   The information is updated when you change the pointer position.

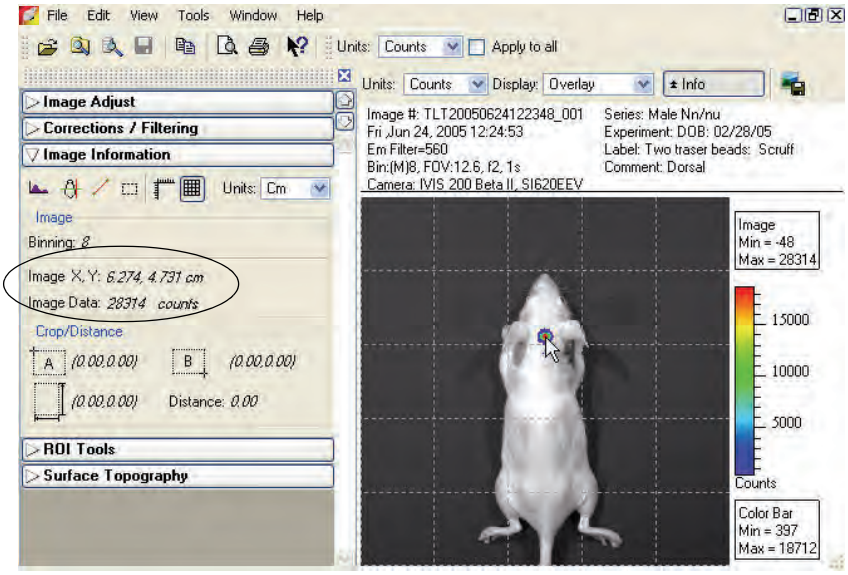


Figure 4.20 Position the mouse arrow in the image window to display x,y coordinates and intensity data

Viewing an Image Histogram

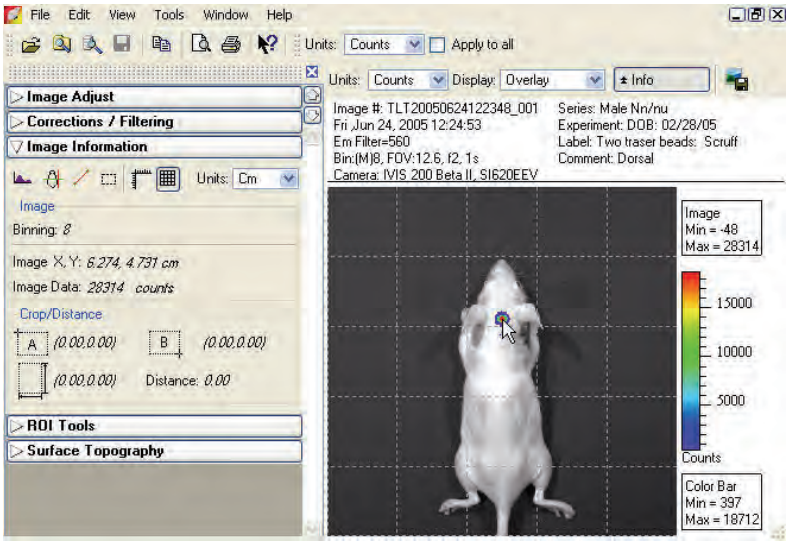
The image histogram plots a frequency distribution of the pixel intensities in an image (Figure 4.21). The software sorts the intensities into groups (*bins*) and plots the number of pixels per bin.

NOTE

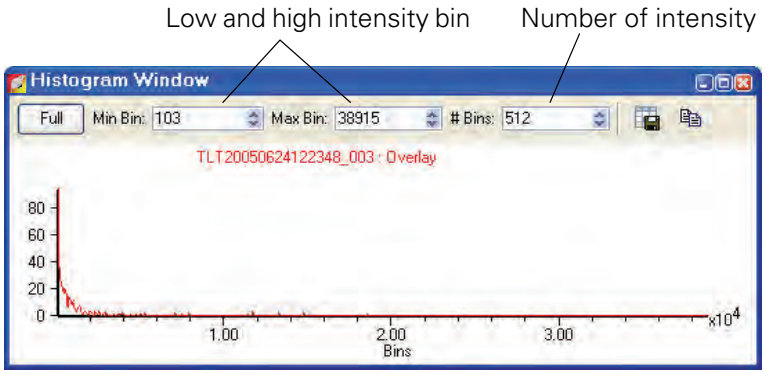
In the Overlay display mode, the histogram plots the luminescent data. To obtain a histogram of the photograph, select Photograph from the Display drop-down list.

To display the image histogram:

1. Open the image of interest.
2. In the Image Information tools, click the **Image Histogram** button .
   - The histogram is displayed.
3. To display the histogram using the full intensity range of the image, click **Full** in the Histogram window.
4. To edit the minimum or maximum bin intensity, enter a new value in the Min Bin or Max Bin box, or click the arrows.
5. To edit the number of bins, enter a new value in the # Bins box or click the arrows.
   - The histogram is updated.



Note: By default the Auto min/max range of the image data determines the histogram range and bins (the software sets the min and max values to optimize image display and suppress background noise). Click **Full** to use the actual image min/max data to generate the histogram range and bins.



To export the histogram data:

1. Click the **Export** button .
2. In the dialog box that appears, select a directory and enter a file name.
3. Select the file type (.csv or .txt) and click **Save**.

To copy the graph to the system clipboard:

1. Click the **Copy** button .

Figure 4.21 Image histogram
The histogram plots number of pixels (y-axis) per intensity bin (x-axis).

Viewing a Line Profile

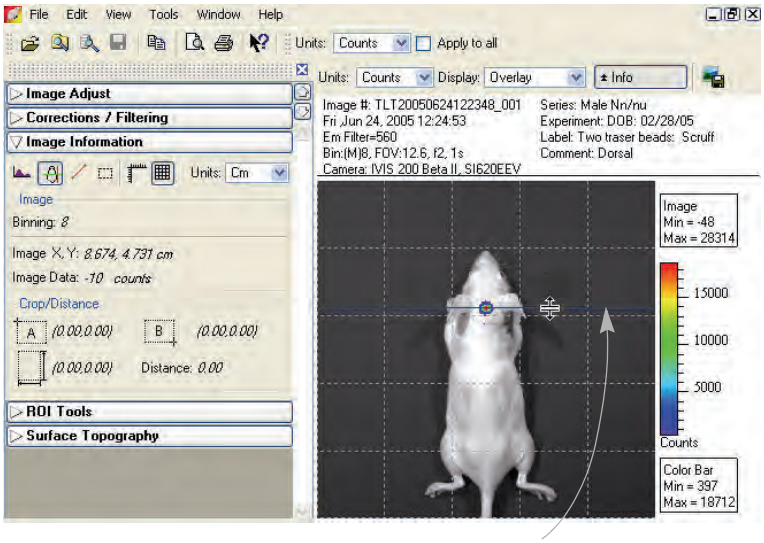
The line profile plots pixel intensity data that you select by placing a horizontal or vertical line on the image. The line profile is automatically updated when you change the line position.

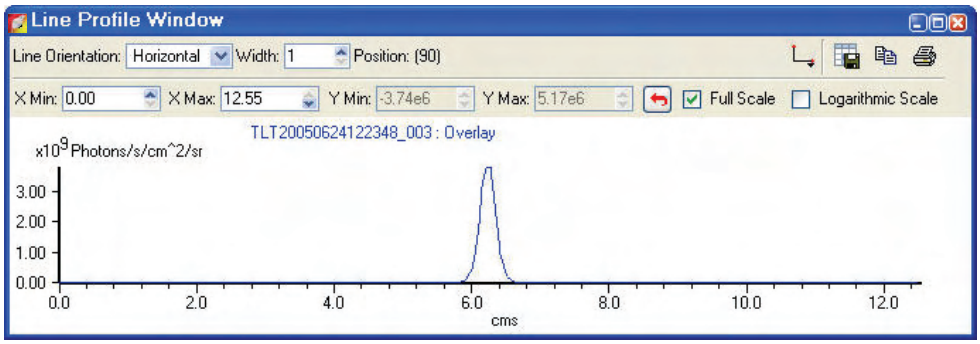
NOTE

In the Overlay display mode, the line profile plots the luminescent data. To obtain a histogram of the photograph, select Photograph from the Display drop-down list.

To display the line profile:

1. Open the image of interest.
2. In the Image Information tools, click the **Line Profile** button.
   - A line appears on the image and the Line Profile window appears.
3. To view the line profile at different locations in the image, put the pointer over the line. When the pointer becomes a ↕, drag the line over the image.
   - The line profile is updated as the line moves over the image.





The blue line determines the pixel intensities that are plotted in the line profile graph. Move the line on the image to update the line graph.

Figure 4.22 Line profile plots intensity (y-axis) at each pixel (x-axis) from a user-specified line in the image

Item in the Line Profile Window	Description
Line Orientation	Choose Vertical or Horizontal from the drop-down list to set the orientation of the line in the image window.
Width	Sets the line width.
Position	Line position (pixels).
X Min X Max	Displays the minimum and maximum value of the x-axis. Use the arrows to change the x-axis min or max. If photons is selected in the image window, the x-axis units = pixels. If counts is selected in the image window, the x-axis units = cm. To display the range available for the Min or Max, place the mouse pointer over the Min or Max edit box.

Item in the Line Profile Window	Description
Y Min Y Max	Displays the minimum and maximum value of the y-axis. Use the arrows to change the y-axis min or max. To display the range available for the Y Min or Y Max, place the mouse pointer over the Min or Max edit box.
	Click to reset the X and Y Min and Max values to the defaults.
Full Scale	Select this option to display the full X and Y-axis scales. If
Logarithmic Scale	Select this option to apply a log scale to the y-axis.
	Click this button to choose a grid line pattern to display in the line profile window.
	Click this button to export the line profile data to a .csv or .txt file.
	Click this button to copy the line profile graph to the system clipboard.
	Click this button to open the Print dialog box.

Making Image Measurements

Figure 4.23 shows how to measure distance on an image.

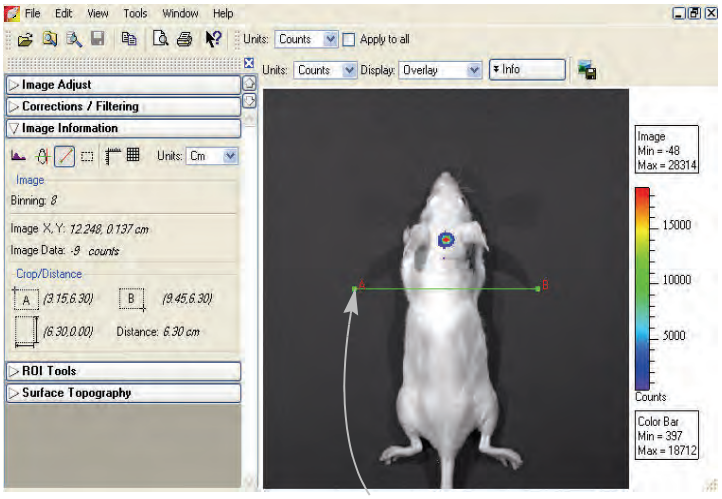
Figure 4.24 shows how to display a measurement box on an image.

To measure distance:

1. Open the image of interest and in the Image Information tools, click the **Distance Measurement Cursors** button .
   - A measurement cursor is displayed on the image. The position and length of the cursor are displayed in the tool palette.
2. To change the cursor position or size, drag the A or B end of the cursor to a new location on the image.
   - The measurement information in the tool palette is updated.

Position and length of the measurement cursor:

	Pixel x,y coordinates of location A on the cursor.
	Pixel x,y coordinates of location B on the cursor.
	Length of the cursor from A to B (number of pixels), vertical distance from A to B (number of pixels).
Distance	Length of the cursor from A to B. (number of pixels).



Measurement cursor

Figure 4.23 Measuring distance with the measurement cursor

To display a crop box on the image:

1. Open the image of interest and in the Image Information tools, click the **Image Crop** button.
   - The pointer changes to a **+**.
2. Draw a rectangle on the area of interest.
3. To change the size or position of the crop box, drag a box corner or side.
4. To delete the crop box from the image, click the button.

Crop box position and dimensions:

A	x,y coordinates at the upper left corner of the box.
B	x,y coordinates of lower right corner of the box.
	Box width and height.
Distance	Length of the diagonal from the upper left to lower right

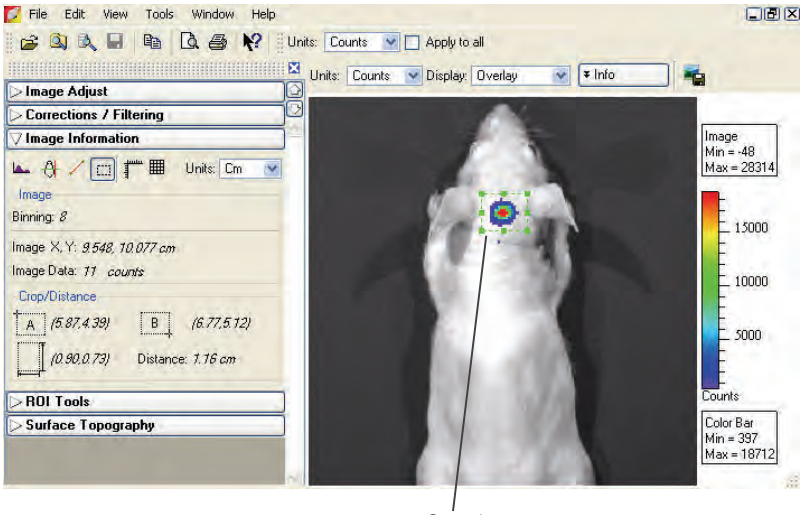


Figure 4.24 Measuring distance with the crop box

4.10 Image Layout Window

The Image Layout window provides a convenient way to annotate and save an image to a graphic file.

- To open the Image Layout window, select **View** → **Image Layout Window** on the menu bar.
- To paste the active image into the Image Layout window, click the button.

Note: If you do not clear the layout before you close the Image Layout window, the same window contents are displayed the next time the window is opened.

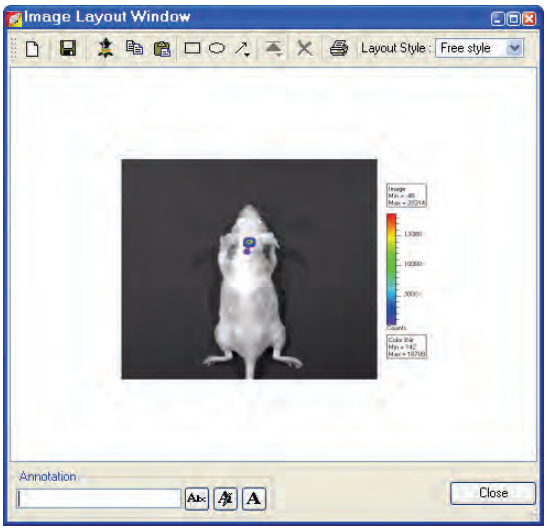


Figure 4.25 Image Layout window

Item in the Image Layout Window	Description
	Clears the Image Layout window.
	Opens a dialog box that enables you to save the Image Layout window contents to a graphic file.
	Pastes the active image in the Image Layout window.
	Copies the contents of the Image Layout window to the system clipboard.
	Pastes the contents of the system clipboard to the Image Layout window.
	Rectangle drawing tool.
	Oval drawing tool.
	Arrow and line drawing tool.
	Select an the item in the Image Layout window. To move the item to the front or back in the window, choose an option from the drop-down list.
	A drop-down list of formatting options for the Image Layout window. For example, the 2x2 layout style provides 4 separate layout areas in the window. A different image can be pasted into each layout area.
	To apply notes to an image, enter text in the annotation box and press **Enter**. Drag the text to the location of interest in the image.
	Opens a dialog box that enables you to select a font or edit the font style and size.
	Opens a color palette that enables you to select a font color or specify a custom font color.
	Opens a text editor that enables you to edit the selected text.

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5 Working With Region of Interest (ROI) Tools

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5.1 About ROIs

A *region of interest* (ROI) is a user-specified area in an image. The ROI tools enable you to create three types of ROIs (Table 5.1).

During a session, the Living Image software records information about the ROIs you create and computes statistical data for the ROI measurements. The ROI Measurements table displays the data and provides a convenient way to review or export ROI information (Figure 5.1). (For more details, see *Managing the ROI Measurements Table*, page 72.)

See Figure 5.1 for a quick guide to drawing measurement ROIs. For more details on ROI drawing methods, see:

ROI Drawing Method	See Page
Manual	60
Automatic	61
Free draw	62

Table 5.1 ROI types

	ROI Types		
	Measurement ROI	**Average Background ROI**	**Subject ROI**
Description	Measures the signal intensity in an area of an image.	Measures the average signal intensity in a user-specified area of the image that is considered background. **Note:** Using this type of ROI is optional. If the animal has significant autoluminescence or autofluorescence, you can determine a background-corrected signal in a measurement ROI by subtracting an average background ROI from a measurement ROI.	Identifies a subject animal in an image. **Note:** Using this type of ROI is optional. It provides a convenient way to automatically associate (link) a measurement and average background ROI for background-corrected ROI measurements when there is significant autoluminescence or autofluorescence.
Available ROI Drawing Methods	• Manual • Automatic • Free draw	• Manual • Free draw	• Manual • Automatic • Free draw
Available Shapes	Circle, square, grid, or contour	Circle or square	Square

5.2 ROI Tools

To display the ROI tools:

1. Open an image.
2. Click ROI Tools in the tool palette.

Note: An image must be active to display the ROI tools. If an image sequence is active, the ROI tools are absent from the tool palette.

ROI tools

Quick guide to drawing measurement ROIs:

1. In the ROI tools, select Measurement ROI from the Type drop-down list.
2. Click the Contour button and select Auto All from the drop-down list.
3. If it is necessary to adjust the ROI boundaries, use the slider or the arrows to change the Threshold % (specifies the minimum per cent of peak pixel intensity that a pixel must have to be included in an ROI identified by the software).
4. Click the Measure button.
   - The ROI measurements are displayed on the image and the ROI Measurement table appears.

   Note: After ROIs have been created, right-click an ROI to view a shortcut menu of ROI commands (**Ctrl**-click for Macintosh users). The shortcut menu provides easy access to many functions for managing ROIs and viewing ROI properties.

The ROI Measurements table displays data for all ROIs created during a session (one ROI per row). The table provides a convenient way to review and export ROI data. For more details on the table, see *Managing the ROI Measurements Table*, page 72.

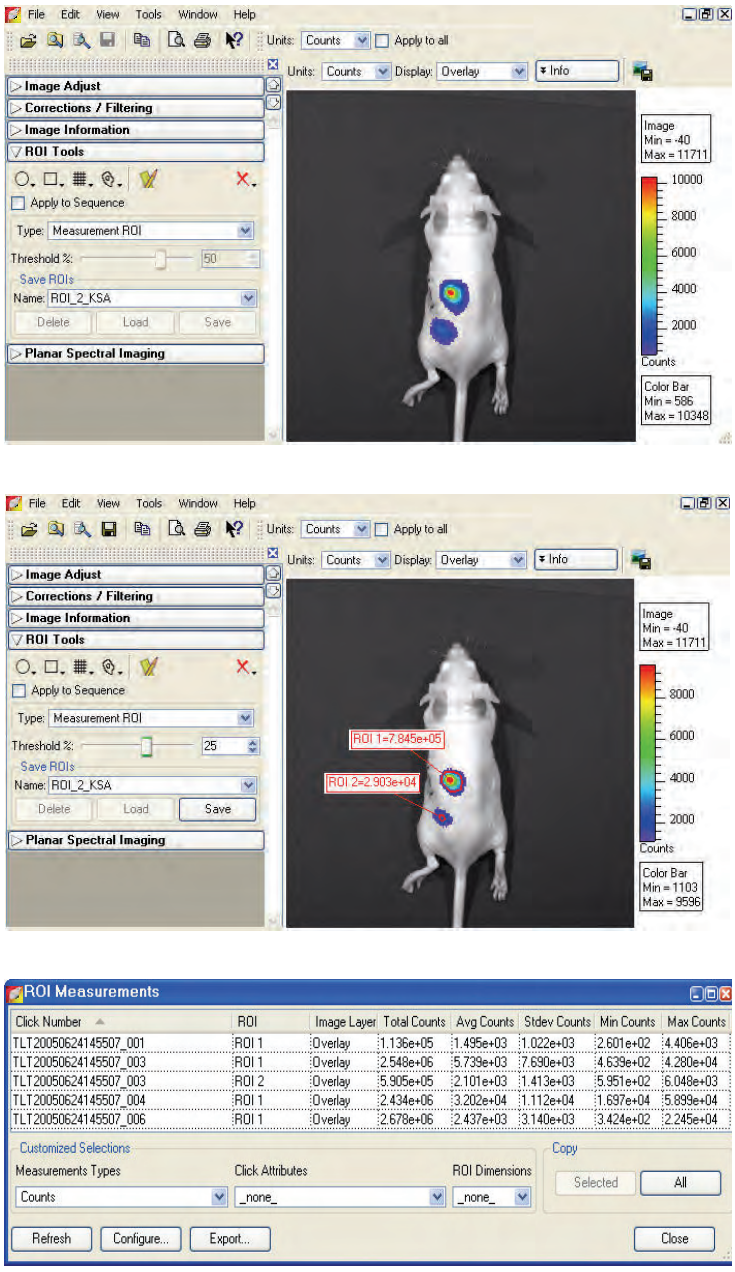

File Edit View Tools Window Help
Units: Counts Apply to all
Display: Overlay Info
Image Adjust
Corrections / Filtering
Image Information
ROI Tools
Apply to Sequence
Type: Measurement ROI
Threshold %: 50
Save ROIs
Name: ROI_2_KSA
Delete Load Save
Planar Spectral Imaging
Image Min = -40 Max = 11711
10000 8000 6000 4000 2000
Counts
Color Bar Min = 586 Max = 10348
Threshold %: 25
ROI 1=7.845e+05
ROI 2=2.903e+04
Color Bar Min = 1103 Max = 9596
ROI Measurements
Click Number ROI Image Layer Total Counts Avg Counts Stdev Counts Min Counts Max Counts
TLT20050624145507_001 ROI 1 Overlay 1.136e+05 1.495e+03 1.022e+03 2.601e+02 4.406e+03
TLT20050624145507_003 ROI 1 Overlay 2.548e+06 5.739e+03 7.690e+03 4.639e+02 4.280e+04
TLT20050624145507_003 ROI 2 Overlay 5.905e+05 2.101e+03 1.413e+03 5.951e+02 6.048e+03
TLT20050624145507_004 ROI 1 Overlay 2.434e+06 3.202e+04 1.112e+04 1.697e+04 5.899e+04
TLT20050624145507_006 ROI 1 Overlay 2.678e+06 2.437e+03 3.140e+03 3.424e+02 2.245e+04
Customized Selections
Measurements Types Click Attributes ROI Dimensions
Counts _none_ _none_
Copy
Selected All
Refresh Configure... Export... Close

Figure 5.1 Displaying the ROI tools and quick guide to drawing ROIs

Item in the ROI Tools	Description
	Click to select the number of circle ROIs to add to the active image.
	Click to select the number of square ROIs to add to the active image.

Item in the ROI Tools	Description
	Click to specify the grid pattern for a measurement ROI that you want to add to the active image. This tool is useful for an image of a multi-well culture plate or microtiter plate.
	Click and select **Auto All** to automatically draw ROIs in the image using the auto ROI parameters. Click and select **Auto 1** to automatically draw one ROI around a user-specified area. For more details on using the auto ROI features, see Figure 5.3, page 61.
	Click to display the ROI Measurements table.
	Click to display a drop-down list of options to delete an ROI(s) in the active image. For more details, see *Deleting ROIs*, page 71. **Note:** These commands do not delete the ROIs that are saved to the system (listed in the Menu Name drop-down list).
Apply to Sequence	Choose this option to apply the selected ROI to all images in a sequence.
Type	Choose the ROI type from the drop-down list: Measurement, Average Bkg, or Subject ROI.
Threshold %	If the Auto All or Auto 1 method is selected, the Threshold % specifies the minimum per cent of peak pixel intensity that a pixel must have to be included in an ROI identified by the software.
Save ROIs	
Name	The name of the selected ROI set or the default name for a new ROI set.
Delete	Deletes the selected ROI set from the system. Note: This permanently removes the ROI from the system.
Load	Applies the ROI set selected from the Name drop-down list to the active image.
Save	Saves the ROI set in the active image. **Note:** This is a global save (the ROI is saved to the system) and the ROI set can be loaded onto any image. If you use the **File → Save** commands to save an image that includes an ROI, the ROI is saved with the image only (not a global save) and is not available for loading onto other images. For more details, see *Saving ROIs*, page 70.

5.3 Drawing Measurement ROIs

- To obtain the intensity signal in an ROI, draw a measurement ROI on the image and click the **Measurement** button .

There are three ways to draw measurement ROIs:

- Manual (Figure 5.2, page 60).
- Automatic (Figure 5.3, page 61).
- Free draw (Figure 5.4, page 62).

Manually Drawing Measurement ROIs

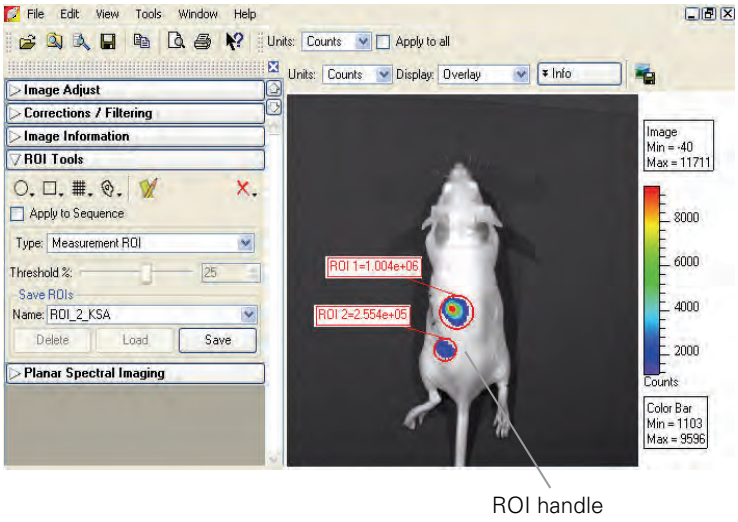
1. Open the image of interest.
   Note: An image must be active to display the ROI tools. If an image sequence is active, the ROI tools are absent from the tool palette.

2. In the ROI tools, select Measurement ROI from the Type drop-down list.

3. If the image is a member of a sequence and you want to apply the ROI to the other images in the sequence, choose the Apply to Sequence option.

4. To specify the ROI shape, click the **Circle**, **Square**, or **Grid** button. On the drop-down list that appears, select the number of ROIs that you want to add to the image or the grid ROI dimensions.
   - The specified type, shape, and number of ROIs are added to the image.

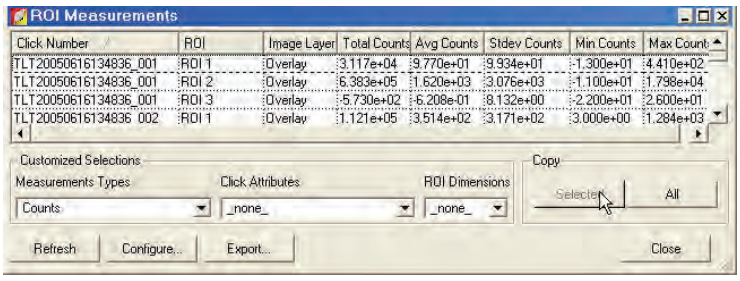
   Note: The grid shape is useful for drawing a grid of ROIs on an image of a microplate plate.



5. To adjust the ROI position:
   a. Place the mouse pointer over the ROI. When the pointer becomes a ✥, click the ROI.
   b. Drag the ROI to the location of interest.

6. To adjust the ROI dimensions:
   a. Place the mouse pointer over the ROI. When the pointer becomes a ✥, click the ROI.
   b. Place the mouse pointer over an ROI handle so that it becomes a ⤡. Drag the handle to resize the ROI.

   Note: You can also change the ROI position or size using the adjustment controls in the ROI Properties box (see *Changing the ROI Position*, page 66 and *Editing ROI Dimensions*, page 67).


ROI Measurements
Click Number ROI Image Layer Total Counts Avg Counts Stdev Counts Min Counts Max Count
TLT20050616134836_001 ROI 1 Overlay 3.117e+04 9.770e+01 9.934e+01 1.300e+01 4.410e+02
TLT20050616134836_001 ROI 2 Overlay 6.383e+05 1.620e+03 3.076e+03 1.100e+01 1.798e+04
TLT20050616134836_001 ROI 3 Overlay 5.730e+02 6.208e-01 8.132e+00 2.200e+01 2.600e+01
TLT20050616134836_002 ROI 1 Overlay 1.121e+05 3.514e+02 3.171e+02 3.000e+00 1.284e+03
Customized Selections
Measurements Types Click Attributes ROI Dimensions
Counts _none_ _none_
Copy
Selected All
Refresh Configure... Export... Close

7. To compute the intensity in the ROI, click the **Measure** button.
   - The ROI label displays the intensity and the ROI Measurements table appears.

Figure 5.2 Manually drawing measurement ROIs

Drawing a Measurement ROI Using the Auto ROI Method

The Living Image software can automatically draw measurement ROIs on an image. To identify ROIs, the software:

- Locates the peak pixel intensities in the image.
- Searches the neighborhood around the peak pixel and includes a pixel in the ROI if the pixel intensity is greater than the *threshold %*, a user-specified percentage of the peak pixel intensity.

To draw measurement ROIs using the Auto ROI method:

1. Open the image of interest (for more details on opening an image, see page 31).
2. In the ROI tools, select Measurement ROI from the Type drop-down list.

   If the image is a member of a sequence and you want to apply the ROI to the other images in the sequence, choose the Apply to Sequence option.
3. To automatically draw all ROIs detected by the software, click an ROI shape button (**Circle**, **Square**, or **Contour**) and select Auto All from the drop-down list.

   - The ROIs appear on the image.

To automatically draw one ROI at a user-specified location:

a. Click an ROI shape button and select Auto 1 from the drop-down list.

   - The create tool appears on the image.

b. Use the ring to move the create tool to the location of interest.

c. Click **Create** on the ring tool.

   - The ROI appears on the image.

4. To measure the signal in the ROI(s), click the Measurement button.

   - The ROI label displays the intensity signal and the ROI Measurements table appears (Figure 5.2).

   For more details on the ROI Measurements table, see page 72.

   Note: For information on how to save ROIs, see page 70.

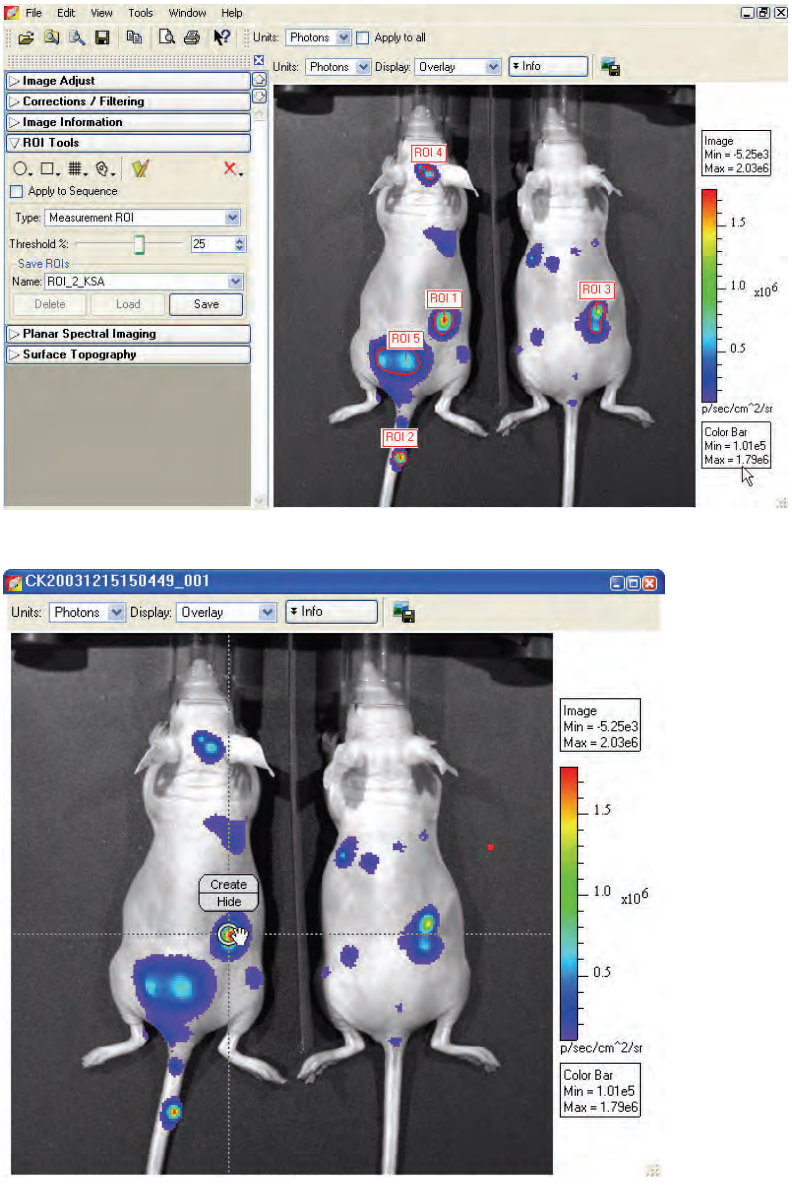


Figure 5.3 Drawing measurement ROIs using the auto ROI method

The software can identify all ROIs on the image that meet the auto ROI parameter thresholds (top) or one ROI at a user-specified location (bottom).

5.4 Drawing an ROI Using the Free Draw Method

To draw an ROI using the free draw method:

1. Open the image of interest (for more details on opening an image, see page 31).
2. Select the type of ROI that you want to draw from the Type drop-down list.
3. Click an ROI shape button (**Circle**, **Square**, or **Contour**) and select Free Draw from the drop-down list.

   Note: The ROI shapes that are available depend on the type of ROI selected.
4. If you selected:

   or - Use the pointer (+) to draw the ROI.

   - Use the pointer (+) to click around the area of interest and draw line segments that define the ROI. Right-click when the last point

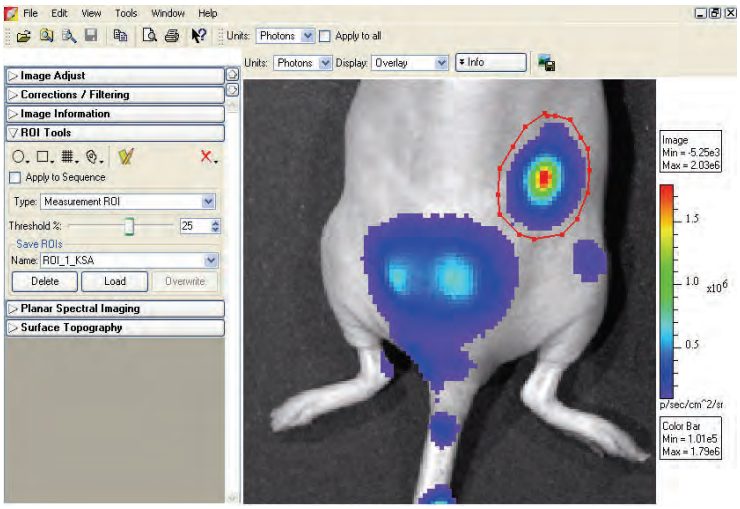


Figure 5.4 Drawing an ROI using the free draw method
In this example, the Contour shape was selected for the free draw method.

5.5 Measuring Background-Corrected Signal

If a subject has significant autoluminescence or autofluorescence, you can obtain a background-corrected ROI measurement by subtracting an average background ROI from a measurement ROI. The software computes:

> Background - corrected intensity signal = Average signal in the measurement ROI - Average signal in the average background ROI

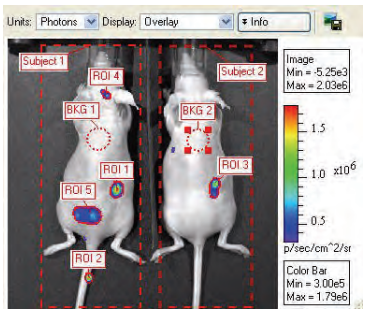
The Image Adjust tools and zoom feature are helpful for selecting an appropriate area for an ROI. By setting the image minimum close to zero and zooming in on a background area in the image, you can determine where naturally occurring background luminescence or autofluorescence is present. For more details on the Image Adjust tools and the zoom feature, see *Adjusting Image Appearance*, page 45 and *Magnifying or Panning in the Image Window*, page 41.

Figure 5.5 shows how to measure background-corrected signal.

To measure background-corrected signal:

1. Draw one or more measurement ROIs on the subject. (For more details, see page 61.)
2. Draw an average background ROI on the subject:
   a. Select Average Bkg ROI from the Type drop-down list.
   b. Click the **Square** or **Circle** button and select **1**.
   - The ROI is added to the image.
   For more details on adjusting the ROI position or dimensions, see page 66 and page 67.
   Note: The average background ROI and measurement ROI do not need to be the same shape or size because the software computes the average intensity signal in each ROI.
3. Use one of the three methods shown below to associate the average background ROI with one or more measurement ROIs.
4. To obtain the background-corrected signal, click the **Measure** button.
   - The ROI label displays the background-corrected intensity and the ROI Measurements table appears. (For more details on the ROI Measurements table, see page 64.)

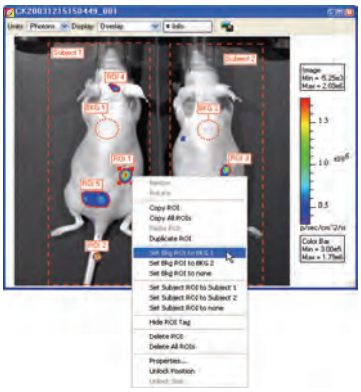
Three ways to associate an avg bkg ROI & measurement ROIs:



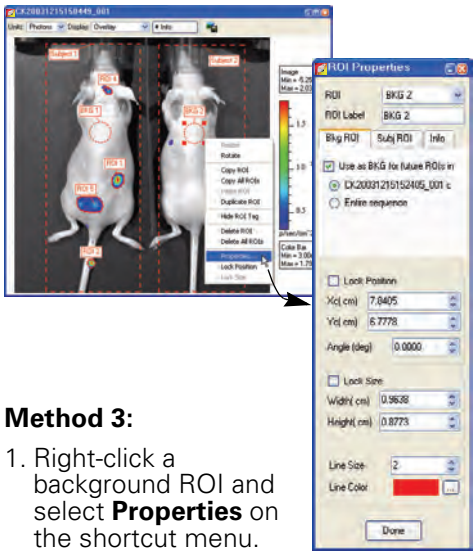
Method 1:
Draw a subject ROI that includes the measurement ROI and the average background ROI.

To draw the subject ROI using the auto ROI feature, select Subject ROI from the Type drop-down list, click the button, and select **Auto All**.

To manually draw the subject ROI, select Subject ROI from the Type drop-down list, click the button, and select **1**. Position the subject ROI so that it includes the measurement ROI(s) and the associated average background ROI.



Method 2:
Right-click the measurement ROI and select **Set BkG ROI to Bkg X** from the shortcut menu that appears.



Method 3:

1. Right-click a background ROI and select **Properties** on the shortcut menu.
2. In the ROI Properties box that appears, click the Bkg ROI tab and put a check mark next to **Use as BKG for future ROIs in**.
3. Choose the image name or the **Entire sequence** option.

ROI Properties

Figure 5.5 Measuring background-corrected signal
Three methods are shown for associating the average background ROI with a measurement ROI(s).

5.6 Managing ROIs

In the ROI Properties box, you can:

- View information about the selected ROI (Figure 5.6, page 64).
- Change the position of the ROI on the image (Figure 5.8, page 66).
- Edit the ROI label and line characteristics (Figure 5.11, page 69).

Viewing ROI Properties

To view ROI properties, do one of the following:

- Double-click the ROI of interest.
- Right-click the ROI and select Properties from shortcut menu that appears.
- Select the ROI, then select **View →Properties** on the menu bar.
  - The ROI Properties box appears.
  Note: The items in the ROI Properties box depend on the type of ROI selected.

To view properties for another ROI, either:

- Click another ROI in the image.
  - The ROI Properties box is automatically updated.
  OR
- Select an ROI from the ROI drop-down list (at the top of the ROI properties box).

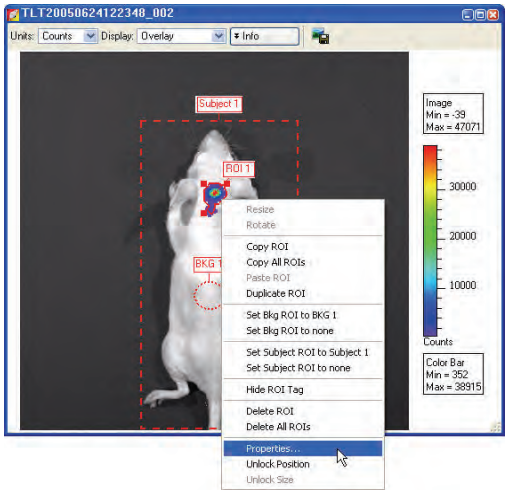


Figure 5.6 ROI properties

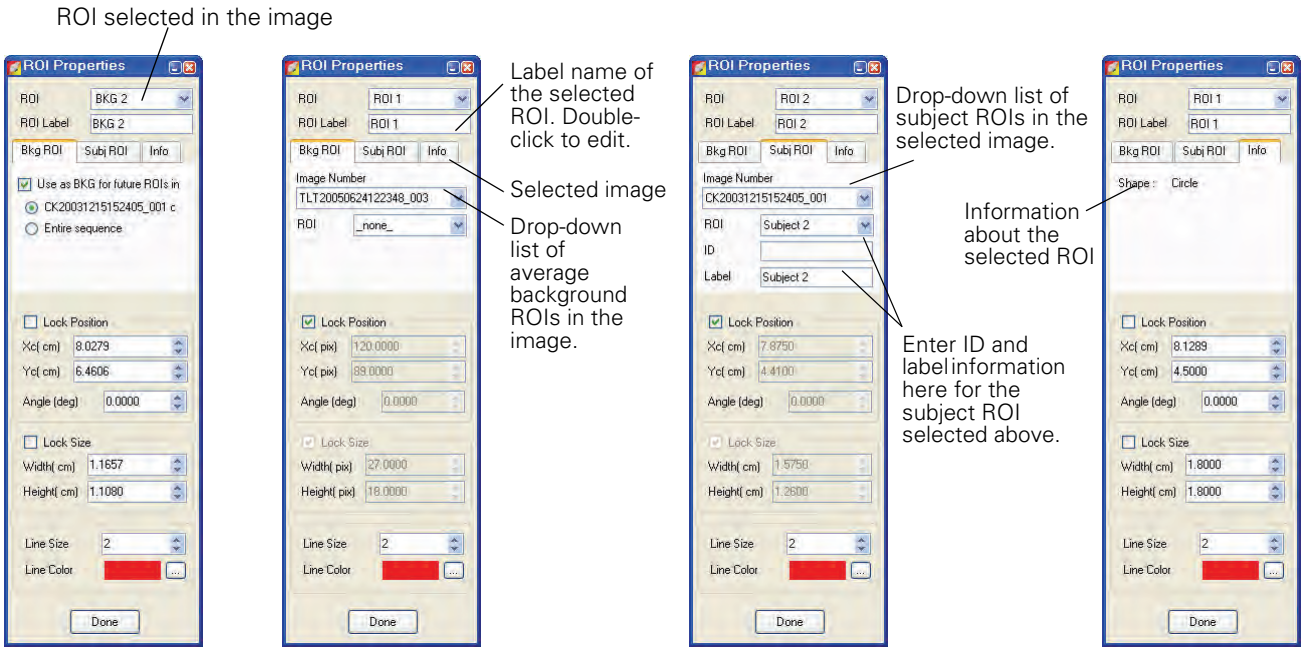

ROI selected in the image
Label name of the selected ROI. Double-click to edit.
Selected image
Drop-down list of average background ROIs in the image.
Drop-down list of subject ROIs in the selected image.
Information about the selected ROI
Enter ID and label information here for the subject ROI selected above.
Bkg ROI tab (average background ROI selected in the image)
Bkg ROI tab (measurement ROI selected in the image)
Subj ROI tab
Info tab

Figure 5.7 ROI properties

Items in the ROI Properties Box	Description
ROI	A drop-down list of ROIs in the active image or image sequence. To select an ROI, double-click the ROI in the image or make a selection from the drop-down list.
ROI Label	Click to edit the selected ROI label name.
Click Number	A drop-down list of open images.
ROI	The Subject ROI tab shows a drop-down list of all subject ROIs in the click number selected above that can be linked to a user-specified measurement ROI or average background ROI (selected from the drop-down list at the top of the dialog box). The Bkg ROI tab shows a drop-down list shows all average background ROIs in the click number selected above that can be linked to a user-specified measurement ROI or subject ROI (selected from the drop-down list at the top of the dialog box).
ID	User-entered information about a subject ROI.
Label	Label name of the selected subject ROI.
Lock Position	Choose this option to lock the position of the ROI selected in the image.
Xc	X-coordinate of the ROI selected in the image.
Yc	Y-coordinate of the ROI selected in the image.
Lock Size	Choose this option to lock the dimensions of the ROI selected in the image.
Width	Width (pixels or cm) of the ROI selected in the image (for more details on setting the units, see *ROI Dimensions*, page 73).
Height	Height (pixels or cm) of the ROI selected in the image.

Items in the ROI Properties Box	Description
Line Size	Specifies the ROI line thickness. To change the line thickness, enter a new value or click the up/down arrows.
Line Color	Specifies the color of the ROI line. To select a line color, click the **Browse** button.
Done	Click to close the ROI Properties box and apply any new settings, including: • Linkages between a measurement ROI and subject ROI (for more details, see *Drawing an ROI Using the Free Draw Method*, page 62). • ROI size dimensions or position • Subject ROI ID information

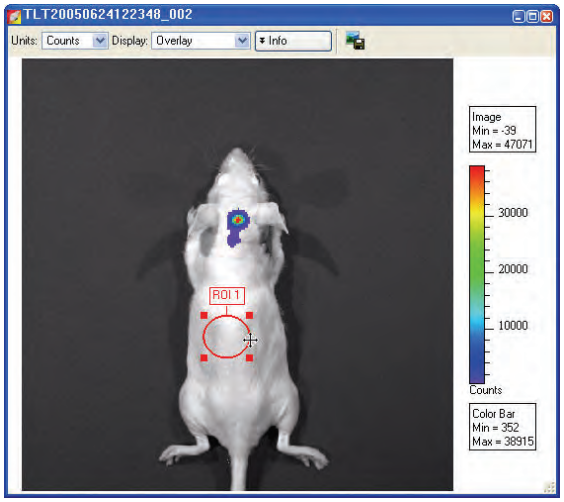
Changing the ROI Position

There are two ways to move an ROI on an image:

- Drag the ROI to a new location.
- Edit the settings in the ROI Properties box (Figure 5.8).

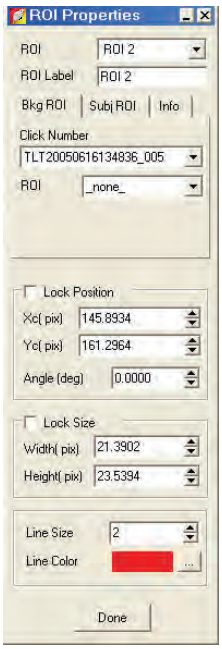
NOTE

You cannot move ROIs created using the auto ROI feature.



To move an ROI using the pointer:

1. Place the pointer over the ROI so that the pointer becomes a arrow.
2. Click and hold the mouse button while you move the ROI.
3. Release the mouse button when the ROI is at the location of interest.



Position, size, line color and line point size of the ROI selected in the image.

To move an ROI using the ROI Properties box:

1. Double-click the ROI in the image.
   - The ROI Properties box appears and displays the positions and dimensions of the selected ROI.
2. To change ROI position, drag the ROI in the image. Alternatively, enter new Xc (x-coordinate) and Yc (y-coordinate) values in the ROI Properties box.
3. To rotate the ROI clockwise, enter the degrees in the Angle (deg) box and click outside the box.
4. To lock the current ROI position, choose the Lock Position option.

   Note: The ROI position cannot be changed until the Lock

Figure 5.8 Moving an ROI on an image

Editing ROI Dimensions

There are two ways to resize a circle or square ROI:

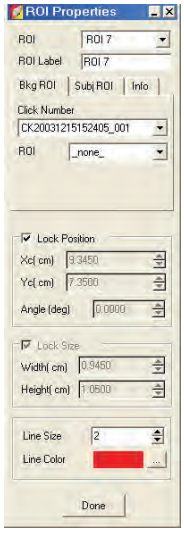
- Drag a handle on the ROI.
- Edit the settings in the ROI Properties box.

NOTE

You cannot change the size of an ROI that was created using the auto ROI or free draw tool.

To resize an ROI using the pointer:

1. Select the ROI and place the pointer over a handle (■)on the ROI.
2. When the pointer becomes a ↘ arrow, drag the handle.

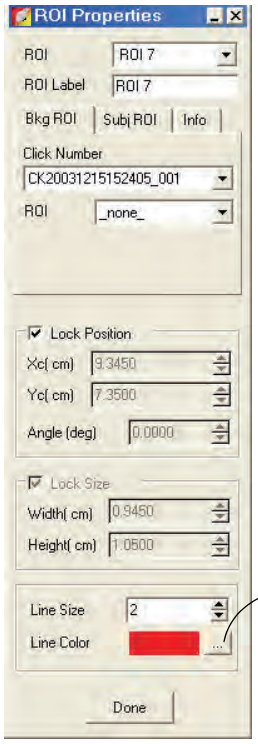


To resize an ROI using the ROI Properties box:

1. Double-click the ROI in the image.
   - The ROI Properties box appears and displays the positions and dimensions of the selected ROI.
2. Enter a new width or height value in the ROI Properties box.
3. To lock the current ROI size, choose the Lock Size option.
   Note: The ROI size cannot be changed until the Lock Size option is cleared.

Figure 5.9 Changing the ROI size

Editing the ROI Line



To edit the ROI line thickness:

1. Enter a new value in the Line Size box. Alternatively, click the arrows.

To change the color of the ROI line:

1. Click the **Browse** button ....
   - The Select Color box appears.
2. To select a basic color for the ROI line, click a basic color swatch, and click **OK**.
3. To specify a custom color, drag the crosshairs in the custom color field, adjust the brightness slider, and click **Add to Custom Colors**.
4. To select a custom color for the ROI line, click a custom color swatch, and click **OK**.

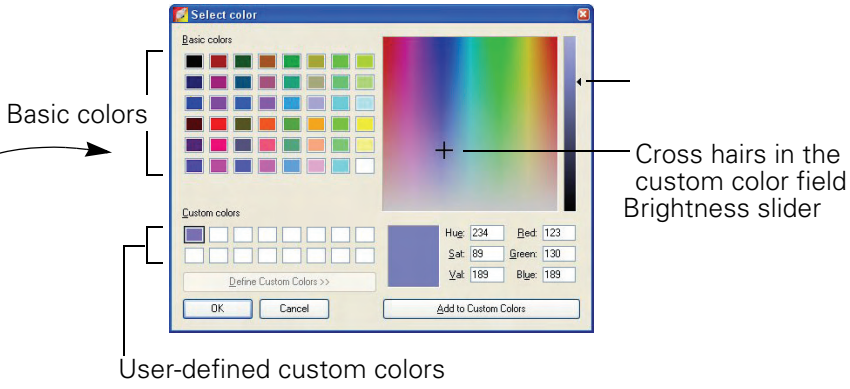
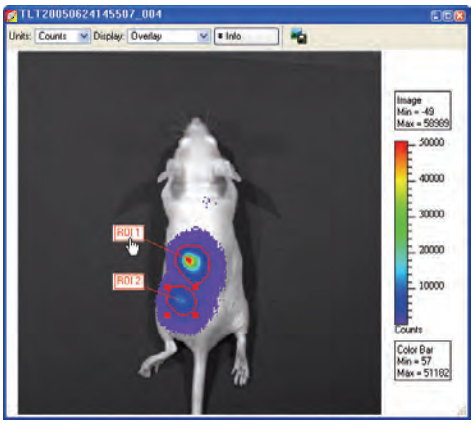


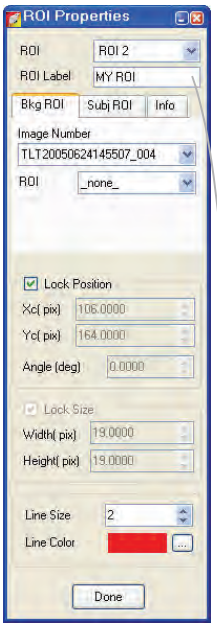
Figure 5.10 Changing the appearance of the ROI

Moving or Editing the ROI Label



To move an ROI label:

1. Place the pointer over the ROI label.
2. When the it becomes a , drag the label.
3. Click to release the label at the new location.



To edit an ROI label:

1. Double-click the ROI. Alternatively, right-click the ROI (**Ctrl**-click for Macintosh users) and select Properties from the shortcut menu.
   - The ROI Properties box appears.
2. Edit the name in the ROI Label box and click **Done**.

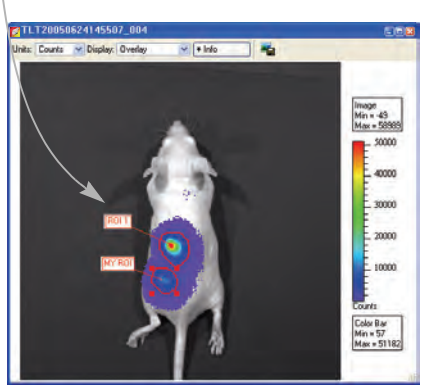
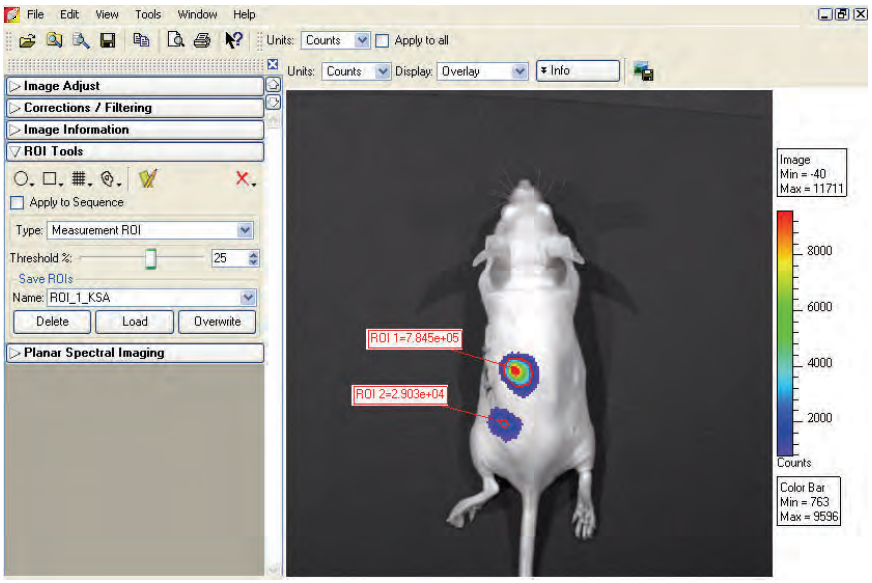


Figure 5.11 Moving or editing an ROI label

Saving ROIs

You can save ROIs with an image and you can also save ROIs to the system (*global save*). ROIs that are saved to the system are available in the ROI Tools and can be applied to other images.



To save the ROI measurements with the image:

1. Close the image.
   Note: If you are working with an image from an open sequence, first close the image, then close the sequence. Click **Yes** in the confirmation message that appears.
   - The ROI measurements are saved to the AnalyzedClickInfo.txt file associated with the image.

To save ROIs to the system:

1. In the Name drop-down list, confirm the default name or enter a new name for the ROI(s).
2. Click **Save**.
   - The ROI(s) from the image are saved to the system and can be selected from the Name drop-down list to apply to other images.

To load ROIs on an image:

1. Open the image of interest.
2. In the ROI tools, make a selection from the Name drop-down list and click **Load**.

Note: If you load ROI(s) onto an image, then draw additional ROIs, the **Save** button changes to **Overwrite**. If you want to save this collection of ROIs using the existing name, click **Overwrite**.

Figure 5.12 Saving ROIs and loading ROIs onto an image

Deleting ROIs

You can delete ROIs from an image or permanently remove ROIs from the system.

To delete ROIs from an image:

1. In the ROI tools, click the button.
2. Make a selection from the drop-down list of delete commands.
   - The specified ROIs are deleted from the image.

   Note: This does not delete ROIs saved to the system (global save).

To permanently remove ROIs from the system:

1. In the ROI Tools, select the ROIs that you want to delete from the Name drop-down list.
2. Click **Delete**.

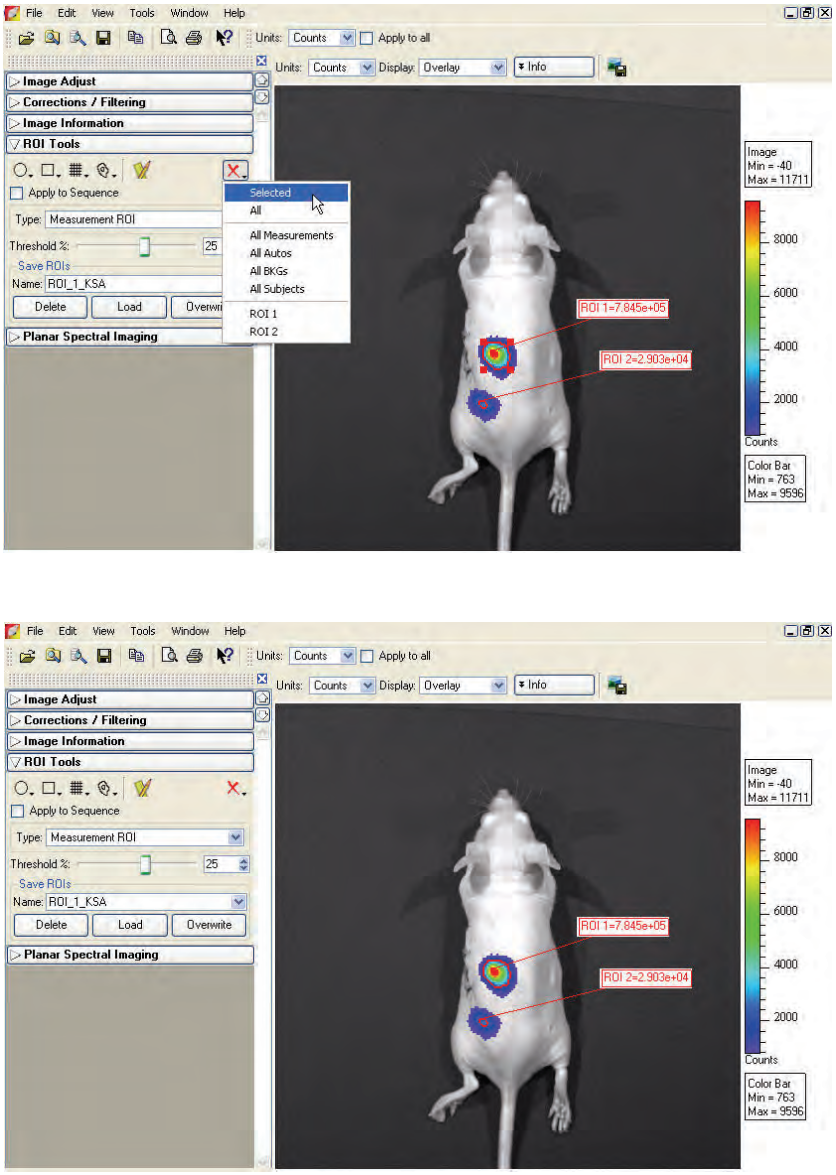


Figure 5.13 How to remove ROIs from an image and delete ROIs from the system

5.7 Managing the ROI Measurements Table

The ROI Measurements table shows information and data for the ROIs created during a session. The ROI measurements can be displayed in units of counts or photons, or in terms of *efficiency*. For more details, see *Quantifying Image Data*, page 151.

To view the ROI Measurements table, click the button. Alternatively, select **View** → **ROI Measurements** on the menu bar.

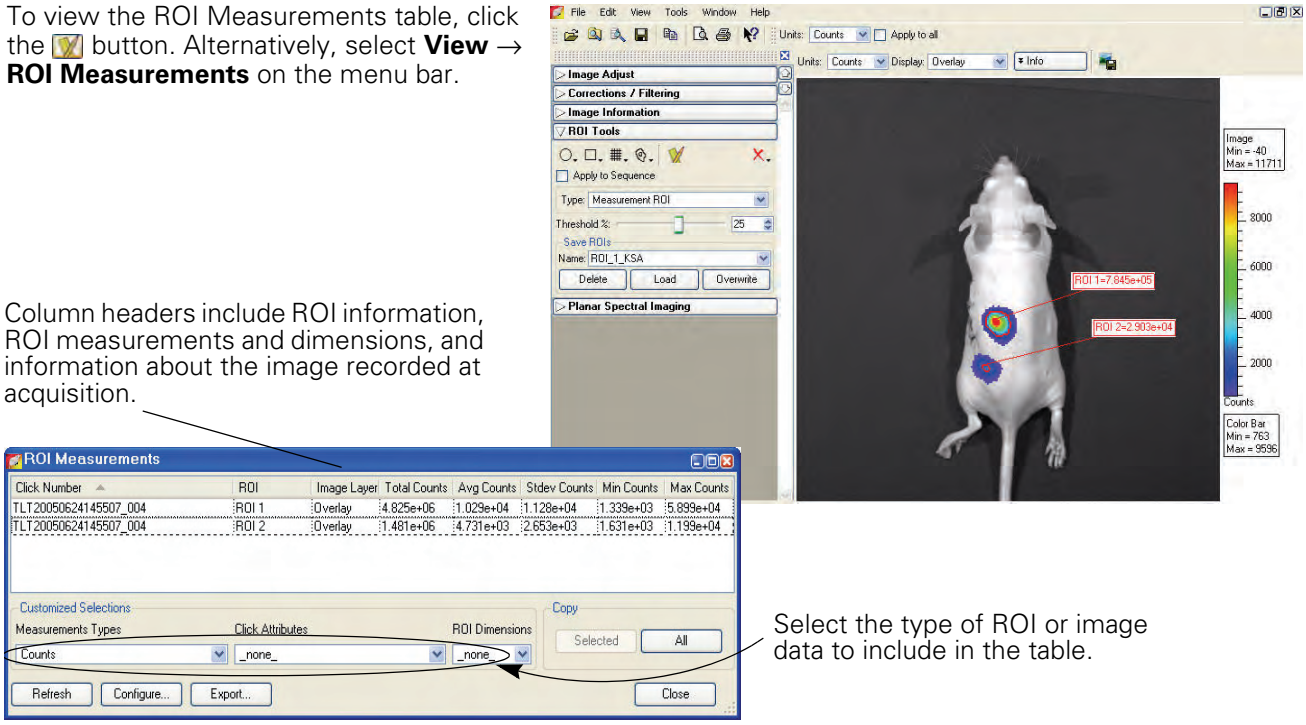


Figure 5.14 Viewing the ROI measurements table

Item in the ROI Measurements Table	Description
Measurement Types	Make a selection from the drop-down list to specify the type of ROI measurements to include in the table.
None	Exclude ROI measurements from the table.
Counts	Includes Total Counts, Avg Counts, Stdev Counts, Min Counts, and Max Counts in the table. Total Counts = the sum of all counts for all pixels inside the ROI. Avg Counts = Total Counts/Number of pixels or super pixels. Stdev Counts = standard deviation of the pixel counts inside the ROI Min Counts = lowest number of counts for a pixel inside the ROI. Max counts = highest number of counts for a pixel inside the ROI. (For more details on count units, see page 151.) Note: These numbers are displayed if the units selected in the ROI Measurements table and the image are the same. Otherwise, N/A appears in each column.

Item in the ROI Measurements Table	Description
Photons	Total Flux = the radiance (photons/sec) in each pixel summed or integrated over the ROI area (cm^2) x 4π. Average Radiance = the sum of the radiance from each pixel inside the ROI/number of pixels or super pixels (photons/sec/cm^2/sr). Stdev Radiance = standard deviation of the pixel radiance inside the ROI Min Radiance = lowest radiance for a pixel inside the ROI. Max Radiance = highest radiance for a pixel inside the ROI. (For more details on photon units, see page 151.)
Efficiency	Available for fluorescent images only. Includes Total Efficiency, Average Efficiency, Stdev Efficiency, Min Efficiency, and Max Efficiency in the table. (For more details on efficiency, see page 151.)
Click Attributes	Make a selection from the drop-down list to specify the click number (image file) information to include in the table. Click attributes include label name settings and camera settings.
None	Excludes click attributes from the table.
All Possible Values	Includes all of the click attributes (label name settings and camera settings) in the table.
All Populated Values	Includes only the click attributes with values in the table.
Xenogen Defaults	Includes all Xenogen Default label name settings in the table.
ROI Dimensions	Make a selection from the drop-down list to specify the ROI dimensions to include in the table.
None	Excludes the ROI area, x,y-coordinates, and dimensions from the table.
Pixels	Includes ROI area, x,y-coordinates, and dimensions (in pixels) in the table.
cm	Includes ROI area, x,y-coordinates, and dimensions (in cm) in the table.
Copy	
Selected	Copies the selected row(s) in the table to the system clipboard.
All	Copies all rows in the table to the system clipboard.
Refresh	Updates the table.
Configure	Displays the Configure Measurements box that enables you to specify and organize the data categories (column headers) for the table.
Export	Displays the Save Measurements box so that the data can be saved to a .txt or .csv file.
Close	Closes the ROI Measurements table.

Configuring the ROI Measurements Table

You can customize the data and information (column headers) in the ROI Measurements table. Several predefined categories are available in the Measurement Types, Click Attributes, and ROI Dimensions drop-down lists.

To configure the ROI Measurements table:

1. Make a selection from the Measurement Types, Click Attributes, or ROI Dimensions drop-down lists.
   - The table is updated with the new data.

To create a custom table configuration:

1. In the ROI Measurements table, click **Configure**.
   - The Configure Measurements box appears. It shows the types of measurements and data in the selected configuration as well as additional items that can be added to the table.
2. Do either of the following:
   - Select a configuration that you want to modify from the User Lists.

   OR
   - Select Customized (Unsaved) from the User Lists.
3. To add an item to the table, select the item of interest in the Available Item list and click **Add**. To remove an item from the table, select the item that you want to remove in the Selected Items list, and click **Remove**.
4. To reorder an item in the Selected Items list, select the item and click **Move Up** or **Move Down**.
   - The columns in the ROI Measurements table are reordered.
5. To save the table configuration, enter a name in the Name box and click **Save**.
   Note: You cannot overwrite a factory loaded configuration. If you modify a factory loaded configuration, save it to a new name.

To delete a custom table configuration

1. Select the configuration from the User Lists drop-down list and click **Delete**.
   Note: Factory loaded table configurations cannot be deleted.

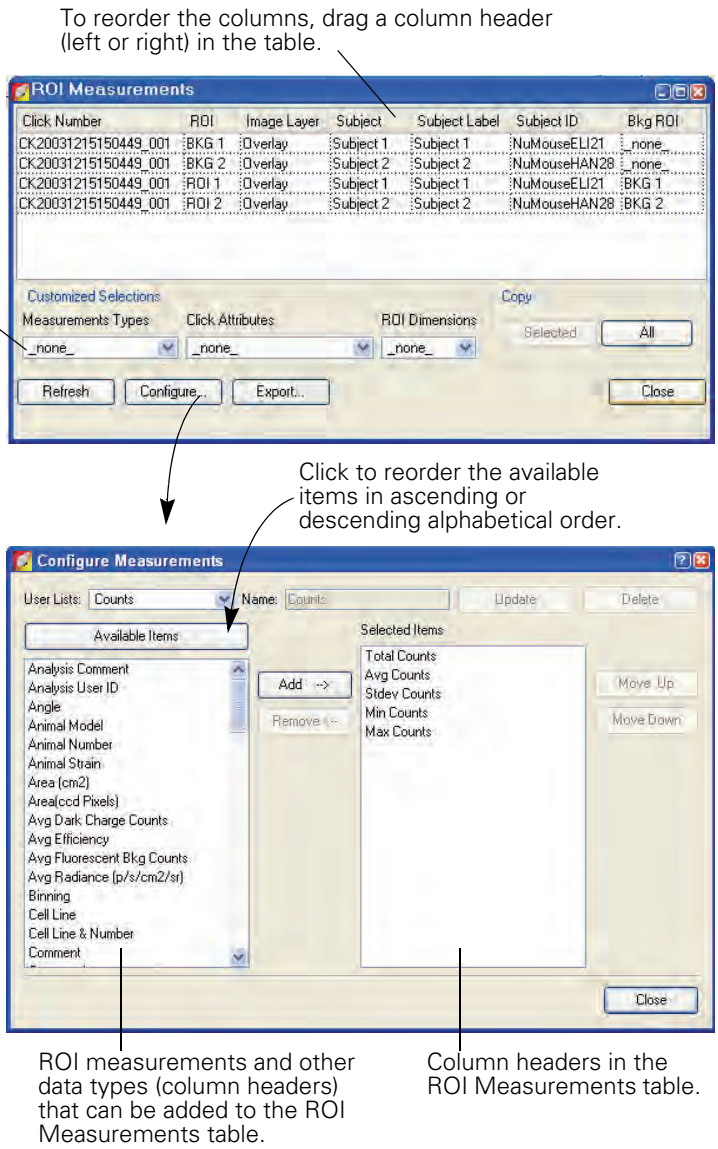
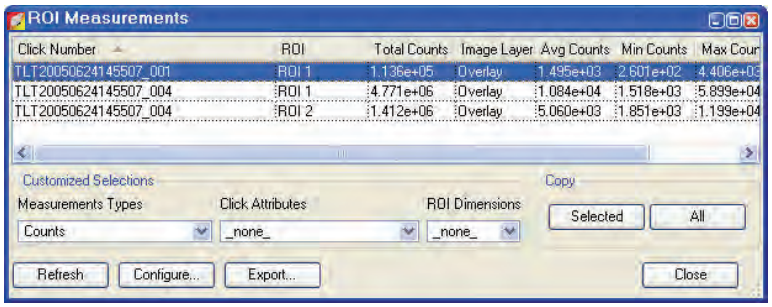


Figure 5.15 Configuring the ROI Measurements table

Copying or Exporting the ROI Measurements Table



To export the table:

1. Click **Export**.
2. In the dialog box that appears, enter a name for the table and click **Save**.
   - The information is saved to a text file (.txt).

To copy:

- Selected rows in the table, select the rows of interest and click **Selected**.
- All rows in the table, click **All**.
  - The information is copied to the system clipboard.

Figure 5.16 Copying or exporting the ROI Measurements table

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6 Performing Image Math Operations

Using Image Math to Create a New Image 78
Subtracting Tissue Autofluorescence 79
Overlaying Multiple Images 82

The Living Image software provides tools that enable you to mathematically combine two images to create a new image.

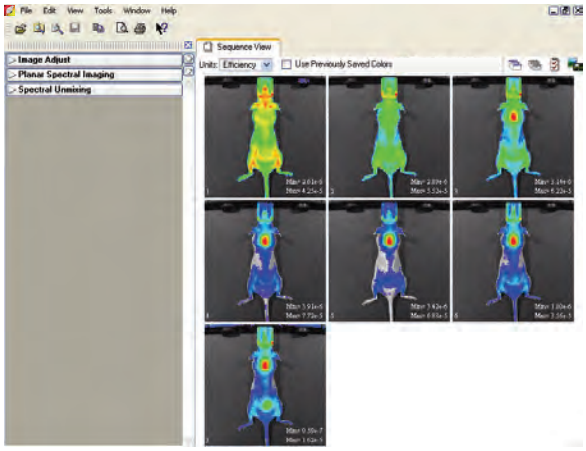
LIving Image Tool	Use This Tool To...	See Page
Image Math	Mathematically combine (add, multiply, subtract, or divide) two user-specified images.	78
Image Math	Remove autofluorescence from a fluorescent image.	79
Image Overlay	Coregister multiple fluorescent or luminescent images on the same photographic image to view multiple reporters in a single image.	83

To perform image math, open an image sequence (see Figure 4.3, page 34) or a group of images (see *Load as Group*, page 32).

6.1 Using Image Math to Create a New Image

To create a new image using image math:

1. Load the image sequence of interest.



2. Select **Tools** →**Image Math for xx_SEQ** on the menu bar.
   - The Image Math window appears.
3. In the Image Math window, select an image of interest from box A and box B.

   Note: For more details on items in the Image Math window, see Table 6.1, 79.
4. Select a mathematical function from the Results drop-down list.
5. To include a scaling factor (k) in the function, enter a value for k.
   - The Image Math window shows a thumbnail of image A, image B, and the new image.

Image Math window

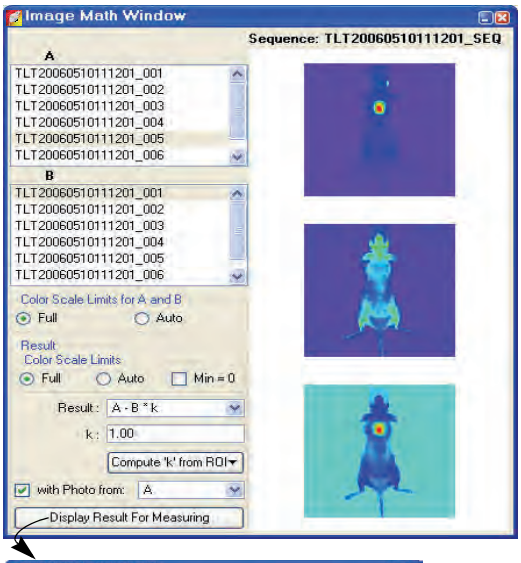


Image selected from box A

Image selected from box B

New image generated by the Result function

New image, overlay mode

To view the new image:

1. Click **Display Result for Measuring**.
   - The mathematical result is displayed.

To save the new image:

1. Click the **Save** button. Alternatively, select **File** →**Save** on the menu bar.
2. In the dialog box that appears, select a directory, and click **Save**.
   - A folder of data is saved to the selected location (AnalyzedClickInfo.txt, ClickInfo.txt, luminescent and photographic TIF images).

To export the new image to a graphic file:

1. Click the **Export** button.

Figure 6.1 Creating a composite image
Image math enables you to create a new image by mathematically combining two images.

Table 6.1 Items in the Image Math window

Item	Description
Color Ranges for A and B	
Full	Choose this option to set the Max and Min values to the maximum and minimum data values in the image.
Auto	When this option is chosen, the software sets the Min and Max values to optimize image display and suppress background noise. The Min and Max settings can be manually adjusted to further optimize the image display for your needs. **Note:** The color bar scale does not affect the image math result.
Color Ranges for Result Image	
Full	See above.
Auto	See above.
Min = 0	Choose this option to set the minimum data value to zero.
Results	Drop-down list of mathematical functions that can be used to generate the new image, including: A - B * k A + B * k A * B * k (A/B) * k
k, Image Math window	A user-specified scaling factor applied in the results function.
k, Fluorescent Background Subtraction window	The software computes k = the ratio of the autofluorescent signal measured using the background filter to the autofluorescent signal measured using the excitation filter in a region on the animal where no fluorophore is present.
Display Result for Measuring	Opens the image generated by image math in an image window.
with Photo from	Choose this option to display the new image in overlay mode using the selected photographic image. (This option is only available if one of the selected images is an overlay.)

6.2 Subtracting Tissue Autofluorescence

To remove tissue autofluorescence from image data, the IVIS® Imaging System implements a subtraction method using blue-shifted background filters that emit light at a shorter wavelength (Table 6.2).

Table 6.2 Emission, excitation, and background filters used to acquire data that can be corrected for tissue autofluorescence

	Emission Filter	Excitation Filter (Primary Image)	Background Filter (Background Image)
Fluorophore	**Passband (nm)**		
GFP	515-575	445-490	410-440
DsRed	575-650	500-550	460-490
Cy5.5	695-770	615-665	580-610
ICG	810-875	710-760	665-695

The objective of using a background filter is to excite the tissue autofluorescence without exciting the fluorophore. To reduce autofluorescence signal in the primary image data, use the image math tool to subtract the background filter image from the primary excitation filter image.

The software computes:

$$\text{Background corrected signal} = (A - B) \times k, \text{ where:}$$

A = primary image (acquired using the excitation filter)

B = background image (acquired using the background filter)

k = (background signal/primary signal)

The background signal is obtained from a measurement ROI that is located in an area where no fluorophore signal is present. The scale factor k accounts for different levels of tissue autofluorescence due to different excitation wavelengths and filter transmission characteristics.

Figure 6.2 explains how to use the image math tool to subtract tissue autofluorescence after you acquire an image sequence that includes a primary and background image. (For more details on acquiring an image sequence, see Chapter 3, page 17.)

For more details on tissue autofluorescence, see Appendix D, page 178

To subtract tissue autofluorescence:

1. Load the image sequence that includes the primary and background fluorescent images.
2. Open either the primary or background image and:
   a. Optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.
   b. Draw a measurement ROI on an area of the animal that represents background signal (area where no fluorophore signal is present).

   Note: You only need to draw the ROI on one of the images. The software copies the ROI to the other image.
3. Select **Tools →Image Math for xx.SEQ** on the menu bar.
   - The Image Math window appears.

   Note: For more details on items in the Image Math window, see Table 6.1, page 79.
4. In the Image Math window, select the primary image in box A. Select the background image in box B.
5. Click Compute 'k' from ROI and select the ROI (created in step 2) from the drop-down list.
   - The background-corrected signal is displayed.
6. To view the mathematical result (overlay mode) in a separate image window, click **Display Result For Measuring**.

   Note: If necessary, use the Color Scale Min and Max sliders in the Image Adjust tools to adjust the image display.

To save the new image:

1. Click the **Save** button. Alternatively, select **File → Save** on the menu bar.
2. In the dialog box that appears, select a directory, and click **Save**.
   - A folder of data is saved to the selected location (AnalyzedClickInfo.txt, ClickInfo.txt, luminescent and photographic TIF images).

To export the new image to a graphic file:

1. Click the **Export** button.
2. In the dialog box that appears, select a directory, enter a file name, and select the file type from the Save as type drop-down list.
3. Click **Save**.

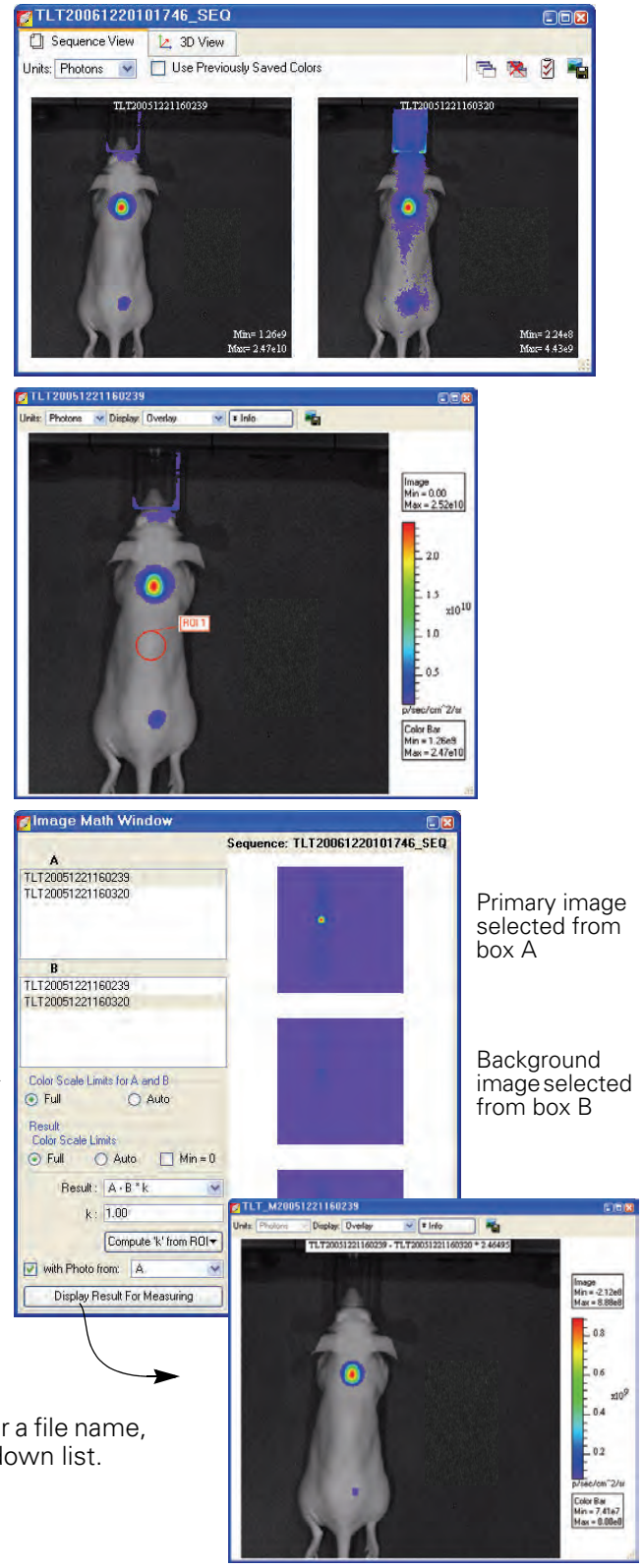


Figure 6.2 Subtracting tissue autofluorescence

6.3 Overlaying Multiple Images

The image overlay tool provides a convenient way to view multiple reporters in one image. You can use the image overlay tool to display multiple luminescent or fluorescent images on one photographic image. The tool provides a convenient way to view multiple reporters in one image. To do this:

- Acquire a fluorescent or luminescent image sequence using the appropriate filters for each reporter. Alternatively, create a sequence from images acquired during different sessions. (For more details, see Chapter 4, page 38.)
- Load the sequence of interest in the Image Overlay window (Figure 6.3).
- Select a photographic image.
- Select the fluorescent or luminescent images that you want to coregister, and specify a different color table for each image (Figure 6.4).

To coregister multiple images:

1. Open the image sequence of interest.

   Note: To view all images in the sequence, click the **Display All** button to open each image (overlay mode) in a separate image window.

2. Open one of the images and optimize the image display using the color scale Min and Max sliders in the Image Adjust tools.

3. Select **Tools→Image Overlay for xx_SEQ** on the menu bar.

   - The image overlay window appears and shows the first photographic image in the sequence.

   (continued in Figure 6.4)

Figure 6.3 Coregistering multiple images (continued in Figure 6.4)

To coregister images (continued from Figure 6.3):

4. Select a photographic image in the upper box.

5. To select all of the fluorescent or luminescent images in the lower box, click the button.

   Alternatively, to select images of interest, do either of the following:

   - To select non-adjacent images in the list, press and hold the **Ctrl** key while you click the images.

   or

   - To select adjacent images in the list, press and hold the **Shift** key while you click the first and last image in the selection.

   Note: In the overlay that is generated, the signal in each image is assigned a different color table. The photographic image is at the bottom of the stack and the last fluorescent or luminescent image selected from the list is at the top of the stack.

6. To remove all fluorescent or luminescent images from the overlay, click the button.

7. To change the display of a fluorescent or luminescent image in the overlay:

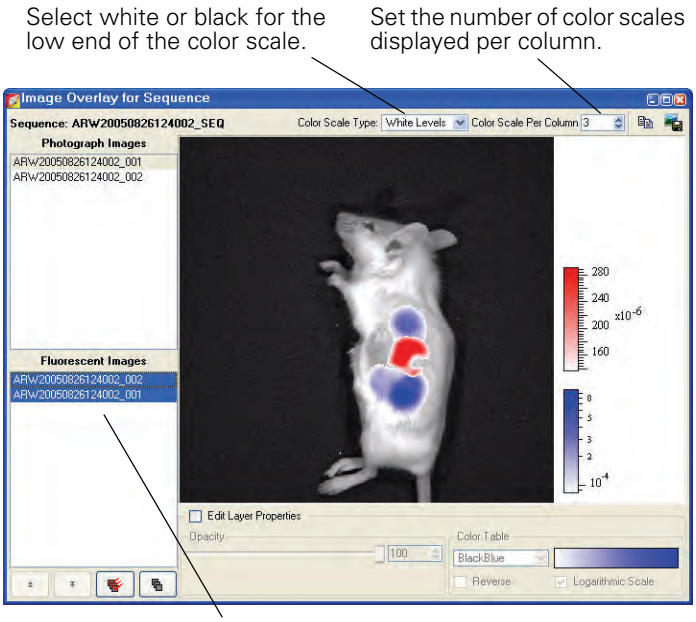
   a. Select the image in the lower box.

   b. Choose the **Edit Layer Properties** option.

   c. Adjust the opacity, select a different color table, or edit the color table properties. When finished, clear the **Edit layer Properties** option.

8. To copy the overlay image to the system clipboard, click the **Copy** button .

9. To export the overlay image to a graphic file, click the **Export** button .



To reorder the images in the list:

1. Choose the **Edit Layer Properties** option.
2. Select an image.
3. Click the or arrows.

Figure 6.4 Creating an overlay image of multiple reporters
The image overlay window displays two different fluorescent signals on one photographic image.

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7 Planar Spectral Image Analysis

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Managing Planar Spectral Imaging Results 91

The Living Image® software enables you to apply planar spectral image analysis to an sequence to determine the average depth and total photon flux of a bioluminescent point source in a user-specified region of interest. For more information on planar spectral image analysis, see Appendix E, page 181.

7.1 Image Sequence Requirements for Planar Spectral Image Analysis

Table 7.1 shows the recommended image sequence for planar spectral image analysis. Analyzing more images usually produces more accurate results. At a minimum, the sequence must include a photographic and luminescent image at the first wavelength and a luminescent image at a second wavelength (560-660 nm).

Table 7.1 Recommended image sequence for planar spectral image analysis on the IVIS Imaging System 200 Series

Image Type	Emission Filter					
	560	580	600	620	640	660
Photographic	✓	Select the Reuse Photographs option in the control panel.				
Luminescent	✓	✓	✓	✓	✓	✓

NOTE

For details on how to specify a sequential setup and acquire an image sequence, see Chapter 3, page 17.

7.2 Planar Spectral Imaging Tools

To display the planar spectral imaging tools:

1. Open an image sequence. (For more details on opening an image sequence, see page 31).
2. Click **Planar Spectral Imaging** in the tool palette.
   - The planar spectral imaging tools are displayed in three tabs (Figure 7.2).

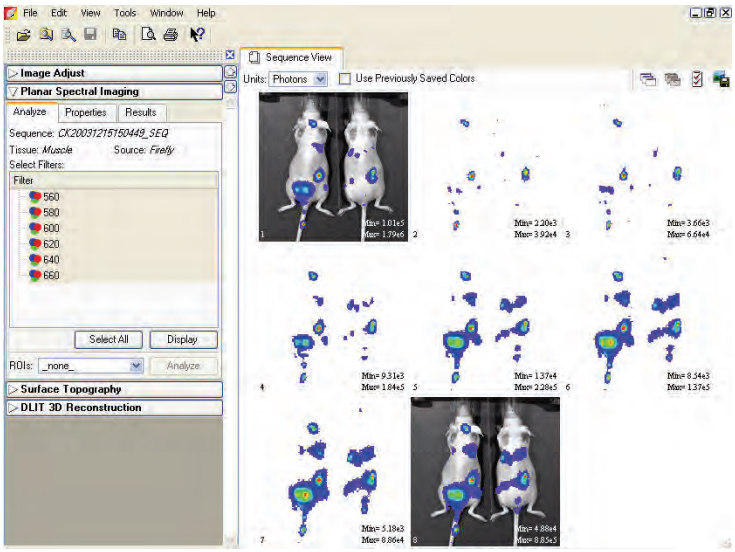
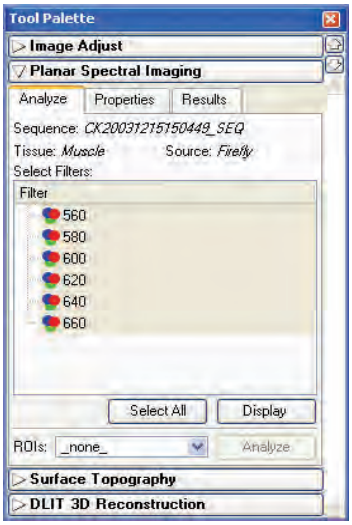


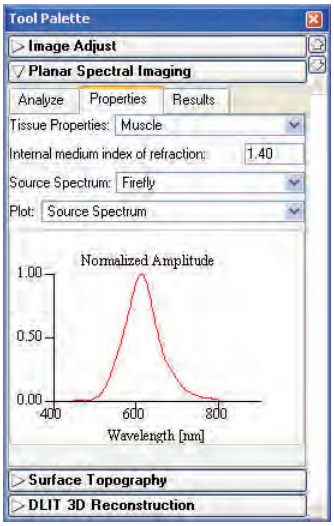
Figure 7.1 Planar spectral imaging tools

Tissue & source selected in the Properties tab

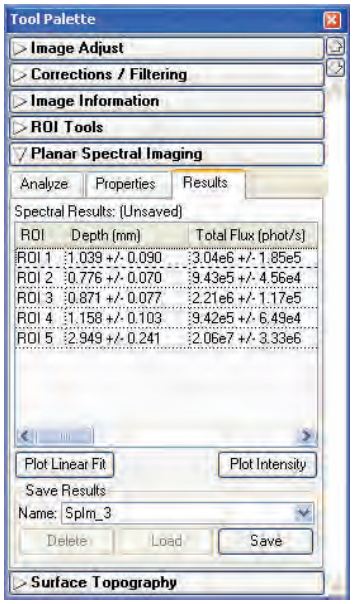
Active image sequence



Analyze tab



Properties tab


Tool Palette
Image Adjust
Corrections / Filtering
Image Information
ROI Tools
Planar Spectral Imaging
Analyze Properties Results
Spectral Results: (Unsaved)
ROI Depth (mm) Total Flux (phot/s)
ROI 1 1.039 +/- 0.090 3.04e6 +/- 1.85e5
ROI 2 0.776 +/- 0.070 9.43e5 +/- 4.56e4
ROI 3 0.871 +/- 0.077 2.21e6 +/- 1.17e5
ROI 4 1.158 +/- 0.103 9.42e5 +/- 6.49e4
ROI 5 2.949 +/- 0.241 2.06e7 +/- 3.33e6
Plot Linear Fit
Plot Intensity
Save Results
Name: Splm_3
Delete Load Save
Surface Topography

Results tab

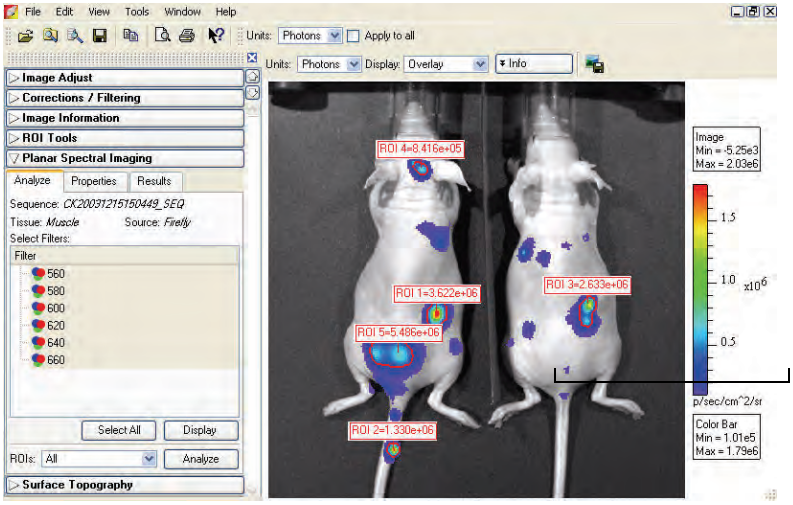
Figure 7.2 Planar spectral imaging tools and results

Item in the Planar Spectral Imaging Tab	Description
Analyze Tab	
Sequence	Drop-down list of open image sequences.
Select Filters	Click the acquisition wavelengths for the images in the selected sequence. To select non-sequential wavelengths, press and hold the **Ctrl** key while you click the wavelengths. (Macintosh users, press and hold the **Cmd** key while you click the wavelengths.)
Select All	Click to select all wavelengths.
Display	Click to display the images from the sequence acquired at the selected wavelength(s).
ROIs	A drop-down list of the ROIs in the active image.
Analyze	Click to perform the spectral analysis.
Properties Tab	
Tissue Properties	Drop-down list of the absorption and scattering properties of various tissues.
Internal medium index of refraction	Tissue index of refraction that is automatically specified when you select a tissue property.
Source Spectrum	Drop-down list of bioluminescent sources.
Display	
Tissue Properties	Click to display graphs (cm^{-1} vs nm) of the absorption coefficient (μ_a), effective attenuation coefficient (μ_{eff}), and reduced scattering coefficient (μ'_s).
Source Spectrum	Click to display the spectrum of the selected bioluminescent source (intensity versus wavelength, normalized to one).
Results Tab	
Spectral Results	
ROI	Name of the analyzed ROI.
Depth	Estimated depth of the point source.
Total Flux	Estimated total photon flux from the point source.
Plot Linear Fit	Click to display a graph of normalized intensity versus the effective attenuation coefficient (μ_{eff}, the optical property of the tissue selected in the Tissue Properties drop-down list) along with the linear fit to these data determined by the spectral analysis code.
Plot Intensity	Click to display a graph of normalized intensity versus wavelength. Intensity is normalized by the selected source spectrum and filter transmission properties.
Save Results	
Name	Default name for the analysis results.
Delete	Deletes the selected results.
Load	Opens the selected results.
Save	Saves the analysis results (results name appears in the Name drop-down list).

7.3 Performing Planar Spectral Image Analysis

To perform planar spectral image analysis:

1. Open the image sequence that you want to analyze.



2. Click Planar Spectral Imaging in the tool palette.

3. In the Analyze tab, select the emission filter wavelengths for the analysis:
   - Click **Select All** to choose all wavelengths at which images were acquired.
   - Alternatively, select only particular wavelengths of interest.

   Note: It is recommended that you do not include a wavelength in the analysis if the signal is less than or equal to the autoluminescent background. If autoluminescent background is a concern, you can create a background ROI and link it to the measurement ROI prior to planar spectral analysis. (For more details, see *Drawing an ROI Using the Free Draw Method*, page 62.)

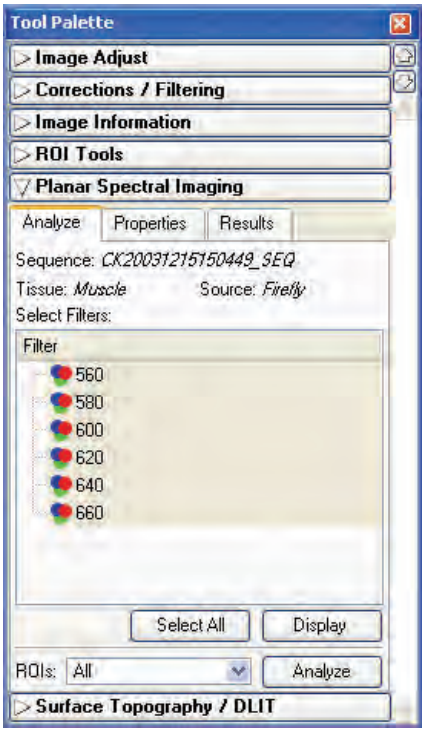
4. Select the ROI(s) for the analysis:
   a. Open the image with the measurement ROI(s) that include the source(s) to be analyzed.
   b. In the ROI drop-down list, select **All** or a particular ROI for the analysis. If there is no measurement ROI, open an image from the sequence and draw an ROI that includes the area for analysis. (For more details on drawing ROIs, see *Drawing Measurement ROIs*, page 59.)

   Note: You only need to draw an ROI on one image in the sequence. The software copies the ROI to all other images of the sequence during the analysis. The ROI should include as much of the light emission from a single source as possible without including too much background.

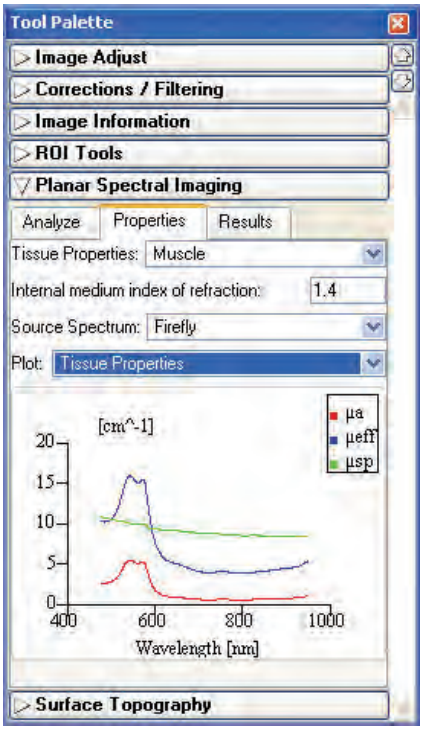
   (continued in Figure 7.4)

Figure 7.3 Performing planar spectral image analysis (continued in Figure 7.4)

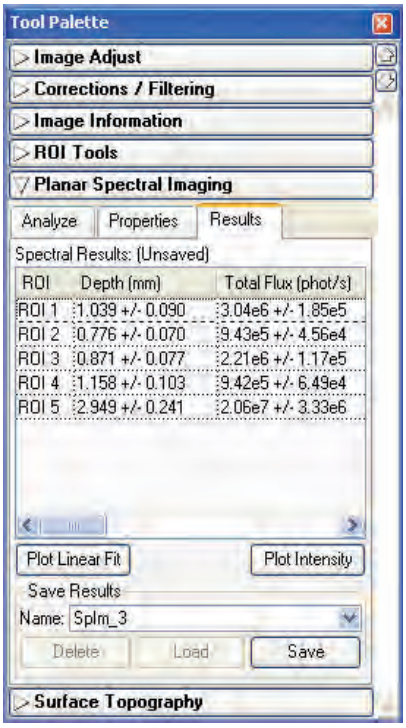
To perform planar spectral image analysis (continued from Figure 7.3):



5. Choose the tissue properties:
   a. In the Properties tab, make a selection from the Tissue Properties drop-down list.
   b. Choose the tissue type most representative of the area of interest. Muscle is a good choice for a generic tissue type.
      - The software automatically sets the internal medium index of refraction based on the selection in the Tissue Properties list.



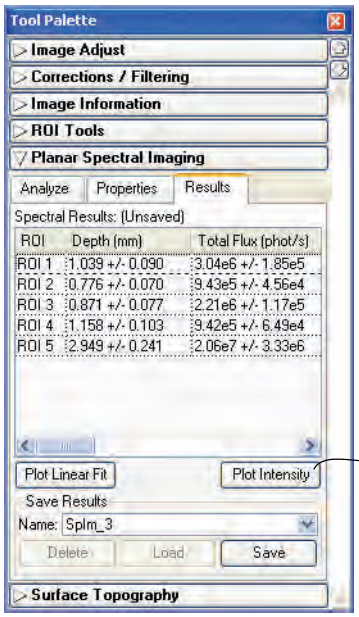
6. In the Properties tab, make a selection from the Source Spectrum drop-down list.
7. Click **Analyze** in the Analyze tab.



The Results tab displays the computed average depth (mm) and total flux (photon/sec) of the bioluminescent point source in the specified ROI(s).

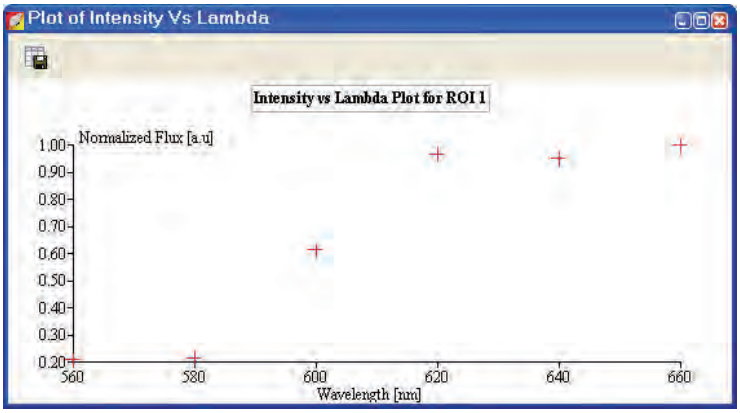
Figure 7.4 Performing planar spectral image analysis (continued from Figure 7.3)

7.4 Viewing & Exporting Graphical Results



To view a graph of the results:

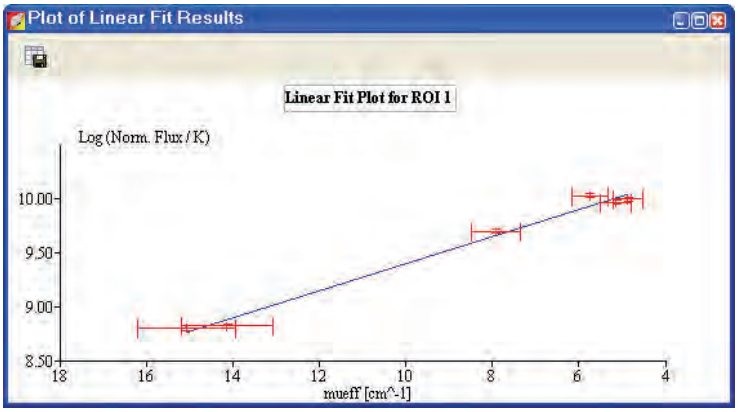
1. In the Results tab, select an ROI.
2. Click **Plot Intensity** or **Plot Linear Fit**.



The intensity graph displays a graph of the measured intensity in the selected ROI at each wavelength in the analysis. The intensity is normalized to the selected source spectrum and the filter transmission properties.

To export graph data to a .csv:

1. Click the **Export Data** button .
2. In the dialog box that appears, select a directory for the data and enter a file name (.csv).
   The data can be opened in a spread sheet application such as Microsoft Excel.



The linear fit graph plots the logarithm of the intensity, normalized to the selected source spectrum and the filter transmission properties, against the optical property of the tissue (μ_{eff}).
The slope of the line is the source depth. If any of the measured points (in red) deviate significantly from the straight line fit, then the analysis results may be suspect.The horizontal error bars represent the uncertainty in the optical properties (usually estimated at ±10%). The vertical error bars represent noise in the image.

Figure 7.5 Viewing graphical results of planar spectral image analysis and exporting the data

7.5 Managing Planar Spectral Imaging Results

To save results:

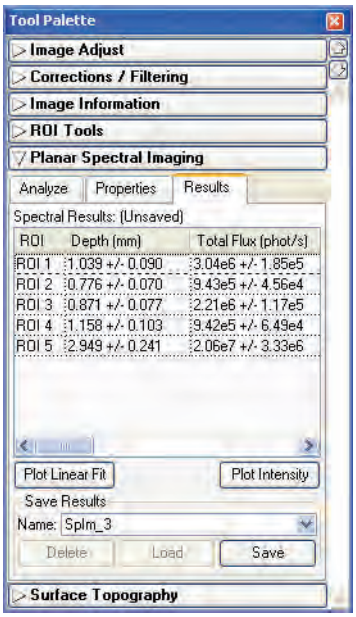
1. Select the results of interest (Splm_xx) from the Name drop-down list
2. Click **Save**.
   - The planar spectral imaging results are saved with the image.

To view results:

1. Select the results of interest from the Name drop-down list.
2. Click **Load**.

To delete results:

1. Select the results that you want to delete from the Name drop-down list.
2. Click **Delete**.



To copy selected results:

1. Right-click the results (row) of interest and select **Copy Selected** from the shortcut menu that appears.
   - The selected results are copied to the system clipboard.

To copy all results:

1. In the Results tab, right-click the results table and select **Copy All** from the shortcut menu that appears.
   - All of the results table is copied to the system clipboard.

To Export Results:

1. Right-click the results table and select **Export Results** from the shortcut menu that appears.
2. In the dialog box that appears, choose a folder for the results, enter a file name (.txt), and click **Save**.

Figure 7.6 Saving and managing planar spectral imaging results

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8 Point Source Fitting

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The point source fitting algorithm is a tool for advanced users that can be used to estimate the optical properties of tissue, the location and power of a point source, or the fluorescent yield of fluorophores. The software analyzes the images in a sequence acquired in one of the following imaging modes:

- Bioluminescence
- Transillumination fluorescence (bottom-illuminated fluorescence)
- Epi-illumination fluorescence (top-illuminated fluorescence)
- Transmission

NOTE

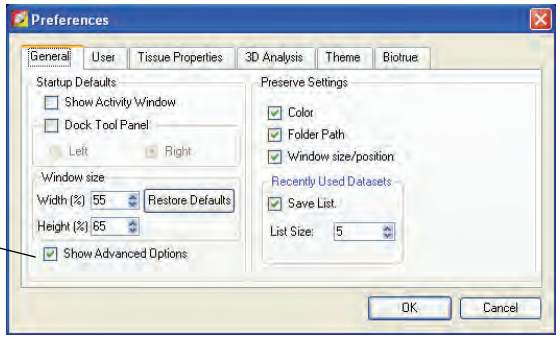
The point source fitting algorithm requires an image sequence that includes one or more images and a structured light image.

8.1 Displaying the Point Source Fitting Tools

The default tool palette does not include the point source fitting tools.

To display the point source fitting tools in the tool palette:

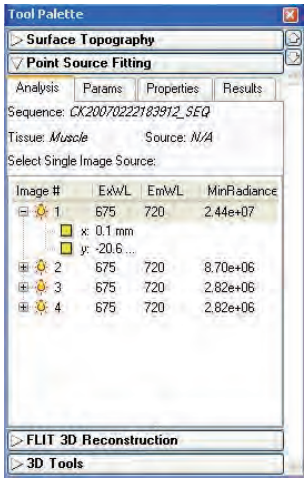
1. Select **Edit →Preferences** on the menu bar.
2. In the dialog box that appears, put a check mark next to Show Advanced Options.
3. Click **OK**.
   - The point source fitting tools appear in the tool palette.



Note: If the image sequence does not include a structure light image, the point source fiting tools do not appear in the tool palette.

Figure 8.1 Preferences dialog box

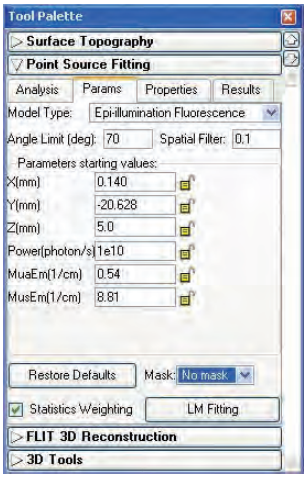
Analyze tab



Analysis tab shows the active image sequence.

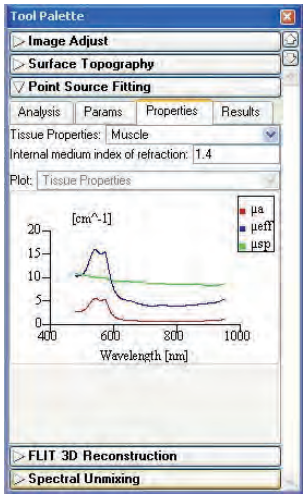
Click the + sign to display the position of the bottom illumination source read from the click info (x,y or x,y,z, depending on the image model).

Params tab



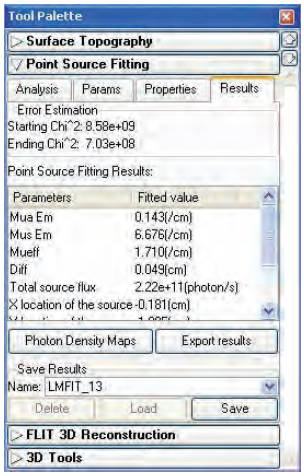
Starting parameter values.

Properties tab



Select other starting values for the optical properties here.

Results tab



Point source fitting results.

Figure 8.2 Tool palette, point source fitting

Items in the Point Source Fitting Tabs	Description
Analysis Tab	
Image #	Image number in the active sequence.
ExWL	Excitation wavelength
EmWL	Emission wavelength
MinRadiance	Minimum surface radiance used for model fitting.
x,y	x- and y-coordinates of the bottom illumination source.
Params Tab	
Model Type	The image acquisition mode.
Angle Limit (deg)	The angle limit refers to the angle between the object surface normal and the optical axis. (For more details, see page 196.)
Spatial Filter	Filters out the noisy data at the mouse edges. A setting of 0.1 means that the analysis includes 90% of the data from the center of mass to the edges.
Parameter starting values	**Note:** Selecting a tissue Properties tab automatically updates MuaEm, MusEm/ MuaEx, and MusEx in the Params tab.
x, y, or z	Source coordinates.
F-yield/Power	Fluorescence yield/strength of illumination or bioluminescence source.
MuaEm	Absorption coefficient at the emission wavelength.
MusEm	Reduced scattering coefficient at the emission wavelength.

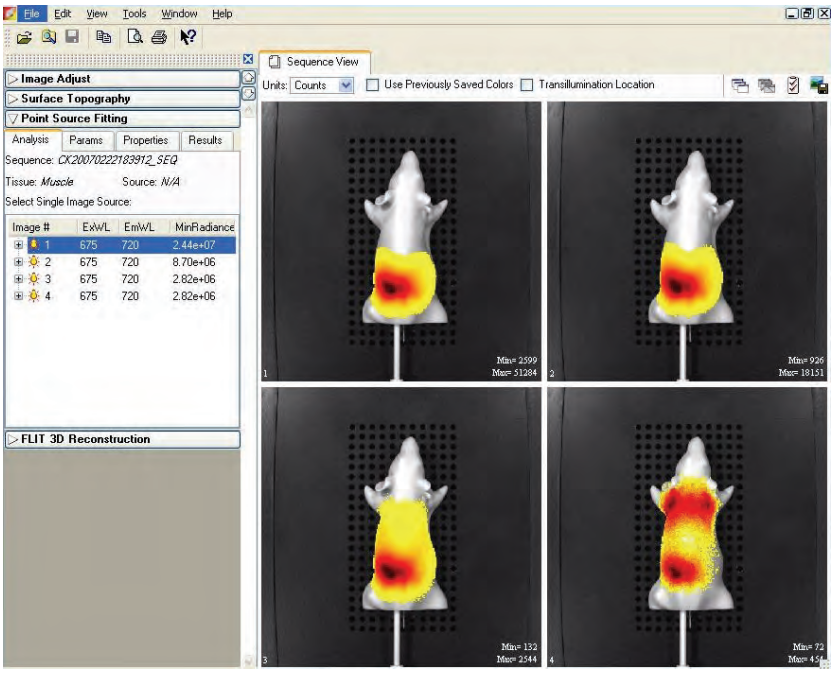
Items in the Point Source Fitting Tabs	Description
MuaEx	Absorption coefficient at the excitation wavelength.
MusEx	Reduced scattering coefficient at the excitation wavelength.
Restore Defaults	Resets the model type, algorithm starting parameters and algorithm options to the default values.
Mask	A drop-down list of ROIs in the selected image. Select an ROI to compute only the source in the ROI.
Statistics Weighting	Choose this option to apply a statistical weighting technique to help reduce the error associated with high radiance measurements.
LM Fitting	Click to begin the point source fitting.
Properties Tab	
Tissue Properties	Make a selection from this drop-down list to specify starting values for the parameters other than the defaults. **Note:** Selecting a tissue property automatically updates MuaEm, MusEm/ MuaEx, and MusEx in the Params tab.
Internal medium index of refraction	The internal medium index of the tissue selected from the Tissue Properties drop-down list. You can also enter a user-specified value.
Results Tab	
MuaEm	Absorption coefficient at the emission wavelength.
MusEx	Reduced scattering coefficient at the excitation wavelength.
Mueff	Effective attenuation coefficient Mueff = $\sqrt{3Mua(Mua+Mus)}$
Diff	Diffusion coefficient, Diff = (Mua +Mus)/3
X location of the source	X-coordinate of the source location.
Y location of the source	Y-coordinate of the source location.
Z location of the source	Z-coordinate of the source location.
Starting ChiSqure	Error between the measured and simulated photon density at the start of the analysis.
Ending ChiSqure	Error between the measured and simulated photon density at the end of the analysis.

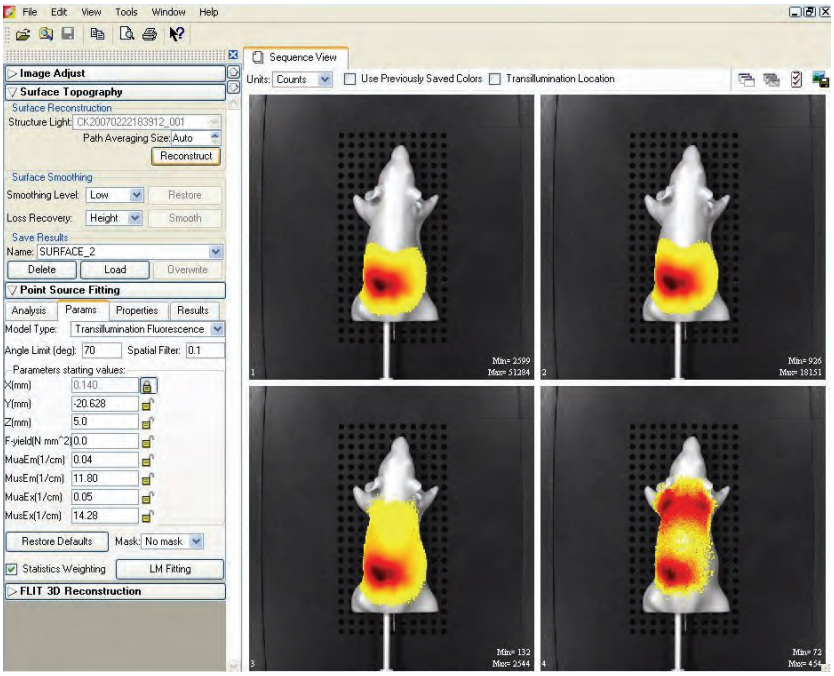
8.2 Performing Point Source Fitting

Point source fitting is performed separately on each image in a sequence.

1. Open the image sequence that you want to analyze.
2. In the Analysis tab, select an image in the sequence.
3. In Surface Topography tools, generate or load a mesh.
   Note: It is recommended that you use the smoothing tool to generate a good quality mesh.
4. Click the Params tab.
   - The default starting values for the source location, power, and tissue optical properties are displayed.

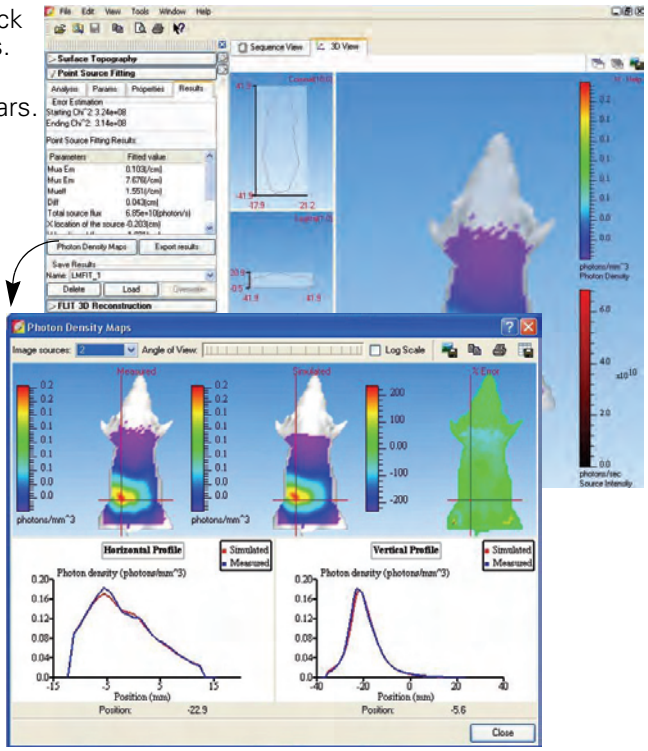
   Note: The software automatically selects the correct model type for the image data.
5. If you want to fix a parameter starting value, click the unlocked icon so that it becomes a closed lock .
6. If you want to construct the source only in a region of interest, make a section from the Mask drop-down list.
7. Confirm the angle limit and spatial filter defaults or enter new values.
8. To specify different starting values for the optical properties:
   a. Click the Properties tab.
   b. Make a selection from the Tissue Properties drop-down list.
   c. Confirm the internal medium index of refraction or enter a new value.
9. In the Params tab, click **LM Fitting**.
   - The source appears on the mesh and the Results tab displays the point source fitting results.





8.3 Checking the Point Source Fitting Results

1. In the Results tab, click Photon Density Maps.
   - The Photon Density Maps window appears.



2. Select the image from the Image sources drop-down list.
3. Compare the simulated and measured photon densities.

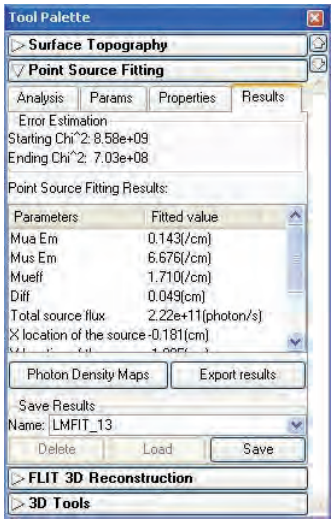
8.4 Exporting Results

To export all results:

1. In the Results tab, click **Export results**.
2. In the dialog box that appears, select the destination folder for the results and click OK.
   - The results include a .txt, .csv, .xsc (source information), and a .xmh (surface mesh) file.

To export user-selected results:

1. Right-click the item of interest in the results list, and select Export Results on the shortcut menu.
2. In the dialog box that appears, choose a folder for the results, enter a file name (.txt), and click **Save**.



8.5 Managing Point Source Fitting Results

To save results:

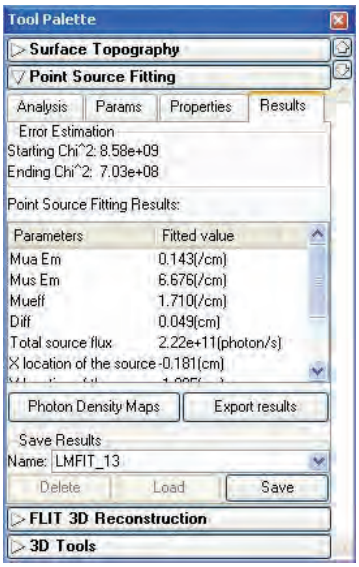
1. Select the results of interest (LMFIT_xx) from the Name drop-down list
2. Click **Save**.
   - The point source fitting results are saved with the image.

To view results:

1. Select the results of interest from the Name drop-down list.
2. Click **Load**.

To delete results:

1. Select the results that you want to delete from the Name drop-down list.
2. Click **Delete**.



To copy selected results:

1. Right-click the results (row) of interest and select **Copy Selected** from the shortcut menu that appears.
   - The selected results are copied to the system clipboard.

To copy all results:

1. In the Results tab, right-click the results table and select **Copy All** from the shortcut menu that appears.
   - All of the results table is copied to the system clipboard.

Figure 8.3 Saving and point source fitting results

9 Spectral Unmixing

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Spectral unmixing removes tissue autofluorescence from fluorescence image data. Spectral unmixing can be applied to images acquired using epi-illumination (excitation light above the stage) or transillumination (excitation light below the stage).

9.1 Performing Spectral Unmixing

It is recommended that the image sequence include several images acquired using the fluorophore excitation filter as well as several emission filters.

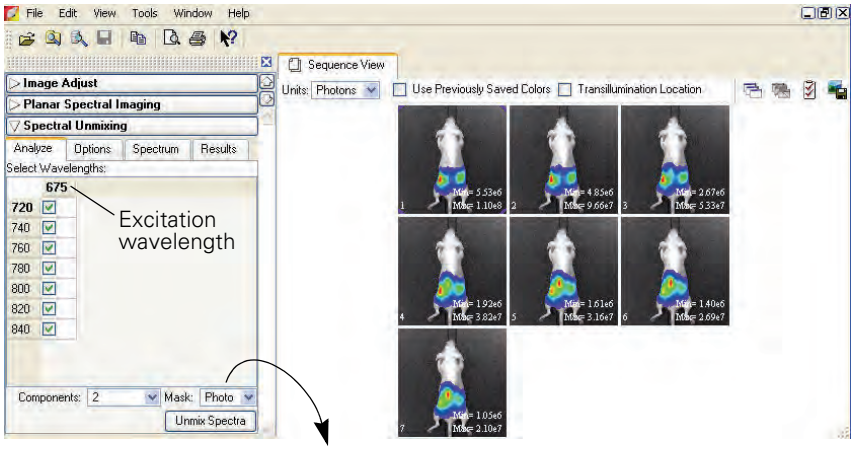
NOTE

Make sure that the band gap between the excitation and emission filters is sufficiently large so that the excitation light does not leak through the emission filter where it can be detected by the CCD.

Figure 9.1 shows how to perform spectral unmixing. In this example, the fluorophore is Quantum Dots 800. Images were acquired using a 675 nm excitation filter and emission filters from 720 to 820 nm in 20 nm increments.

To perform spectral unmixing:

1. Load the image sequence.
2. In the Analyze tab, select the emission wavelengths that you want to include in the analysis.



3. Select the number of spectral components to unmix (the number of fluorophores +1). For example, if the image data includes one fluorophore, then there are two components to unmix: the fluorophore signal and the tissue autofluorescence.
4. Make a selection from the Mask drop-down list:
   - All - The entire image is analyzed.
   - Photo - Opens the Photo Mask Setup window. The purple mask specifies the area for the analysis.
   - ROI - ROIs applied to the sequence are available in the drop-down list. Select an ROI to analyze just the area in the ROI.

   a. Use the Threshold slider or arrows to adjust the mask so that it matches the underlying photograph of the subject as closely as possible without including any area outside the subject image.

   b. Click **Set**.
5. Click **Unmix Spectra**.
   - The spectral unmixing results appear.

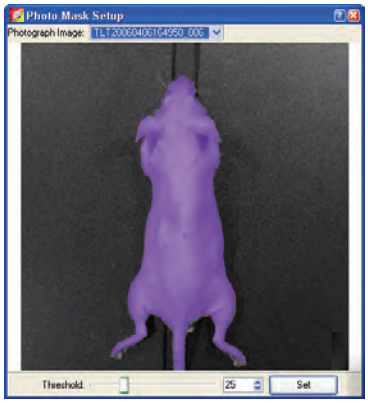


Photo Mask Setup window

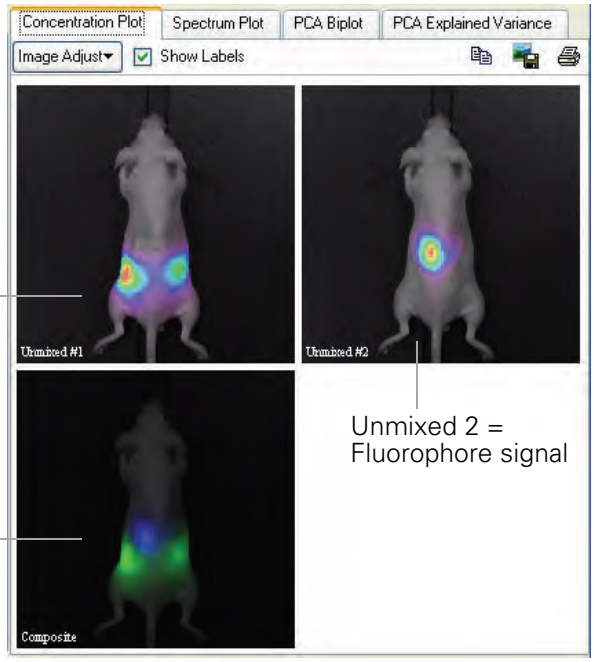


Figure 9.1 Removing tissue autofluorescence by spectral unmixing

9.2 Spectral Unmixing Results Window

The results are displayed in the four tabs of the Spectral Unmixing Results window.

Concentration Plot

The Concentration Plot tab shows a photon density map of each unmixed result and a composite image that includes all of the fluorescent signals, each displayed in a different color.

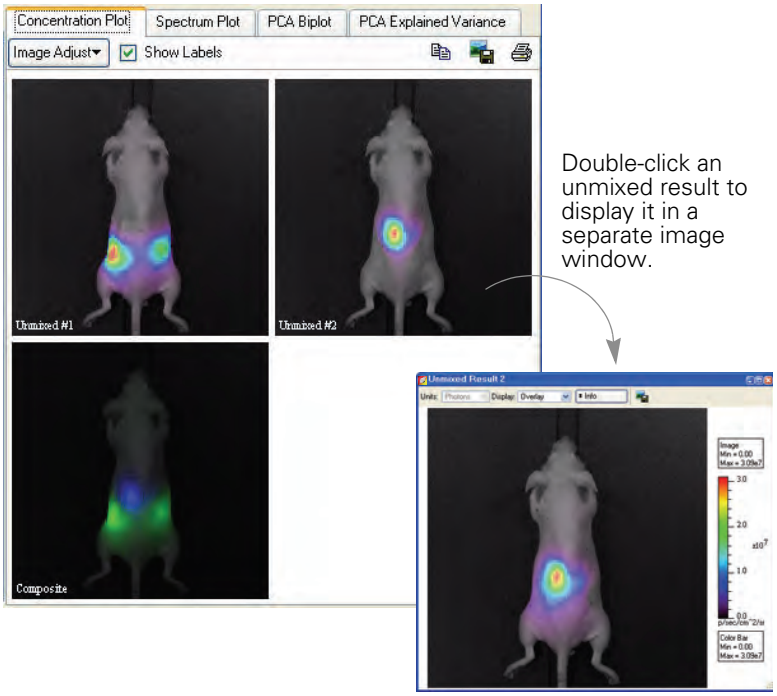


Figure 9.2 Spectral unmixing results, concentration plot

Item in the Concentration Plot	
Image Adjust	Click to show the controls to adjust the fluorescence opacity and the color table display in the concentration plot. Click anywhere in the window to hide the controls.
Show Labels	Choose this option to display image labels on the concentration plot and composite image.

Spectrum Plot

The spectrum plot shows the normalized spectra of the unmixed results. You can edit the appearance of the spectrum plot using the tools in the spectral unmixing tool palette (Spectrum tab) (Figure 9.3).

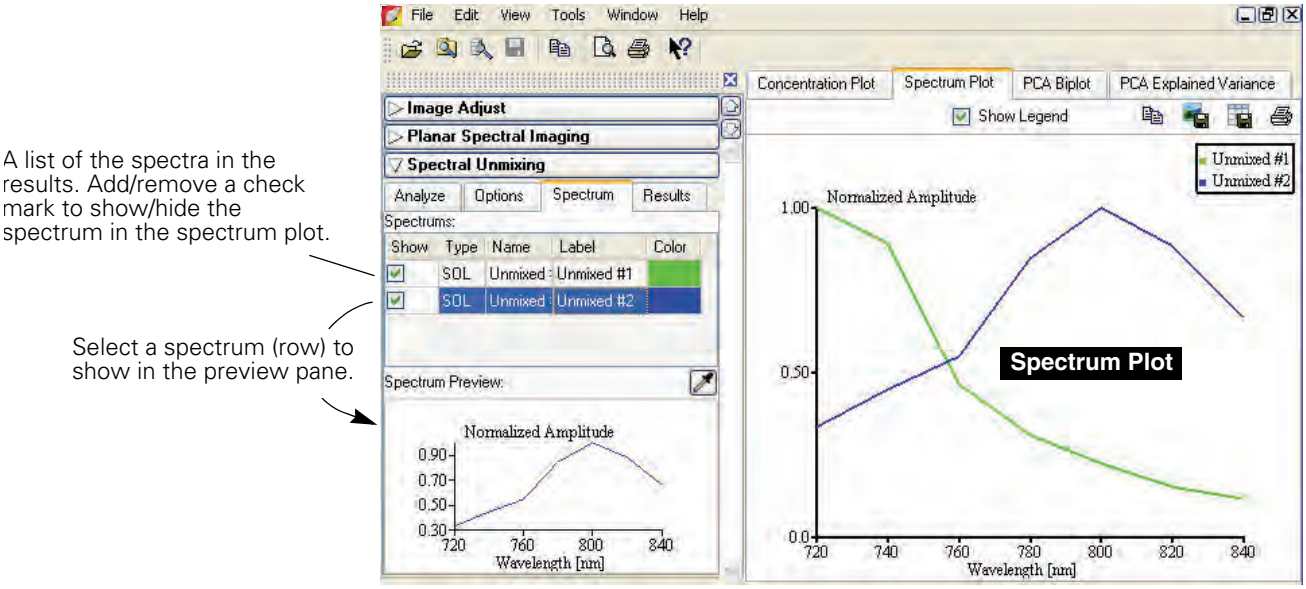


Figure 9.3 Spectral unmixing tools and spectrum plot

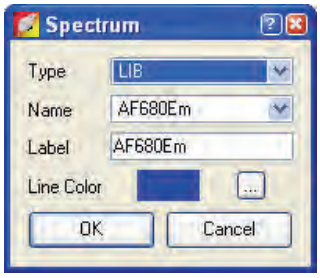
Item in the Spectral Unmixing Tool Palette	Description
Type	The type of spectrum.
SOL	A spectrum generated by the spectral unmixing algorithm.
ROI	A spectrum calculated for a user-selected ROI.
LIB	A user-selected library spectrum. The library includes spectra obtained of different sources obtained using excitation and emission filters.
EXT	A spectrum loaded from the external data provided by the user. This mode is currently disabled.
Name	The spectrum identifier used by the unmixing algorithm. The name cannot be modified.
Label	The spectrum name in the spectrum plot key. The label can be edited.
Color	The color of the spectrum in the spectrum plot. For the SOL type spectrum, it is also the color in the composite image.
Spectrum Preview	Shows the spectrum selected above (click a row above the preview pane).
	Use this tool to pick up a pixel in an opened image and plot the spectrum at this pixel in the spectrum preview.

Modifying the Spectrum Plot

You can modify the appearance of the spectra in the spectrum plot and add or remove spectra from the plot.

To add a spectrum plot:

1. In the spectral unmixing tools, click the Spectrum tab.
2. Right-click the spectrum table and select **Add** on the shortcut menu.
3. In the Spectrum dialog box that appears:



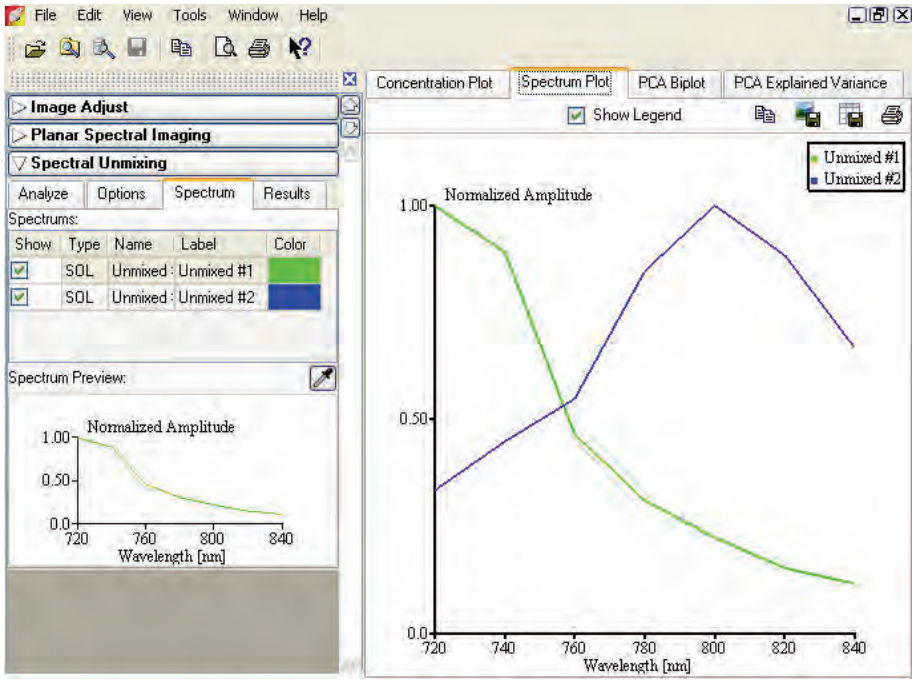
   a. Select the type of spectrum (SOL,LIB, ROI, EXT).

   b. Make a selection from the Name drop-down list.

   c. To select a color for the spectrum graph, click the [...] button.

   d. Click **OK**.

   - The spectrum graph appears in the spectrum plot tab.



To edit a spectrum plot:

1. In the Spectrum tab of the tools, right-click the spectrum (row) that you want to edit.
2. In the Spectrum dialog box that appears, enter a new label or click the [...] button to select a new color for the spectrum graph.
3. Click **OK**.

To remove a spectrum plot:

1. In the spectrum tools tab, right-click the spectrum (row) that you want to delete and select **Delete** on the shortcut menu.

   - The spectrum graph is removed from the plot.

Figure 9.4 Adding, editing, or removing a spectrum plot

PCA Biplot

The PCA biplot is a visualization tool for principal component analysis. It shows a simultaneous display of *n* observations (pixels) and *p* variables (wavelengths) on a two-dimensional diagram.

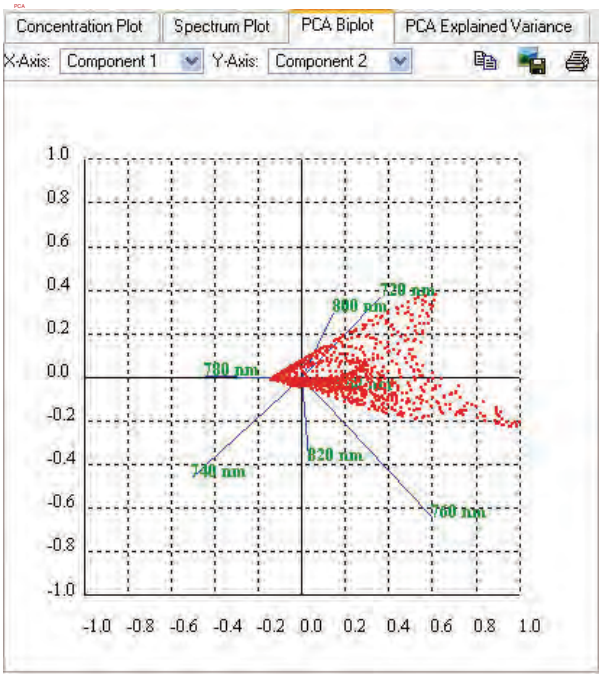


Figure 9.5 PCA biplot

PCA Explained Variance

The PCA Explained Variance histogram shows the part of variance (y-axis) that can be explained by a number of principal components (x-axis).

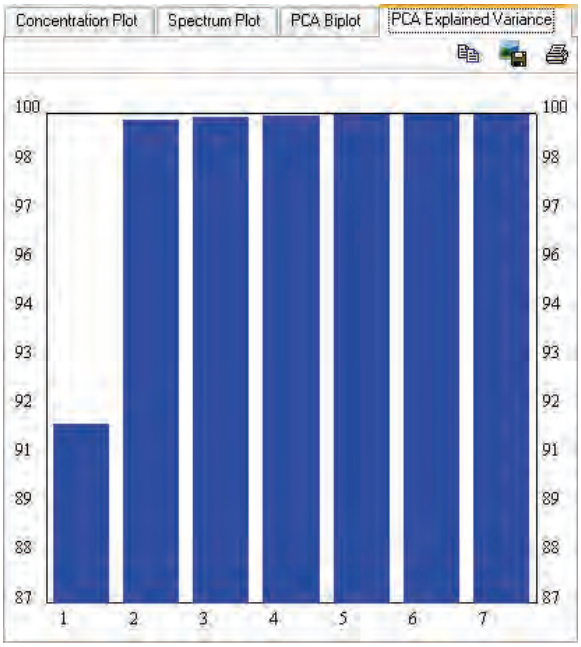


Figure 9.6 PCA explained variance

9.3 Spectral Unmixing Parameters

The Results tab in the Spectral Unmixing tool palette shows the optimized fit parameters used by the software to derive the spectral unmixing results (Figure 9.7).

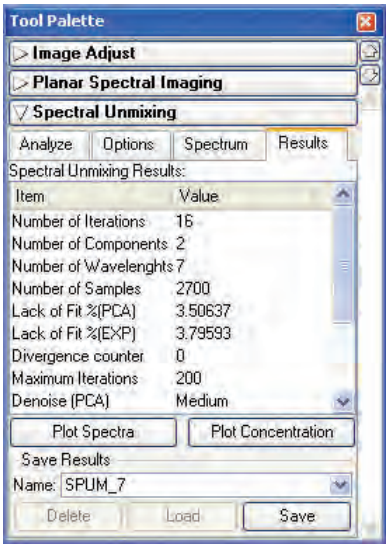


Figure 9.7 Spectral unmixing tools, Results tab

Item in the Results Tab	Description
Number of Iterations	The number of iterations that the algorithm used.
Number of Components	The number of components unmixed.
Number of Wavelengths	The number of wavelength pairs used in the analysis.
Number of Samples	The number of pixel samples used in the analysis
Lack of Fit (%PCA)	The fitting residue compared to the data filtered by principal component analysis.
Lack of Fit (% EXP)	The fitting residue compared to the experimental data.
Divergence Counter	The number of divergences that occurred.
Maximum Iterations	The maximum number of iterations allowed.
Denoise (PCA)	Indicates how much of the data was filtered by principal component analysis.
Normalization	The normalization method used in the analysis.
Non-negativity Method	The non-negativity method used in the analysis.
Weighting Mode	The weighting method applied to the data.
Column Weighting Mode	Indicates if column-wise weighting was used.
Row Weighting Mode	Indicates if row-wise weighting was used.
Plot Spectra	Click to display the spectrum plot tab.
Plot Concentration	Click to display the concentration plot tab.

9.4 Spectral Unmixing Options

The Options tab in the Spectral Unmixing tools, shows the user-modifiable parameters in the spectral unmixing algorithm (Figure 9.8). It is recommended that you first perform spectral unmixing using the default settings. Then, if necessary, change the option settings and reanalyze the data.

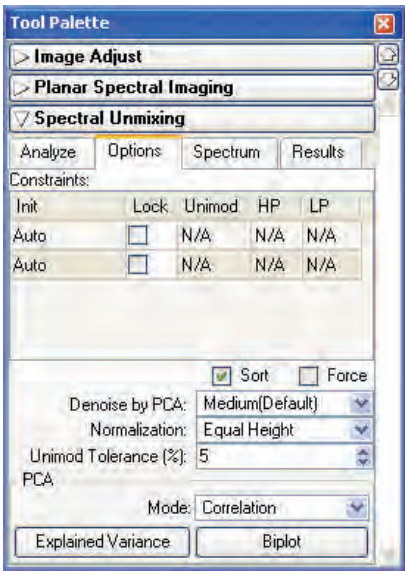


Figure 9.8 Spectral unmixing options

Spectral Unmixing Option	Description
Constraints	The constraints for unmixing the components.
Init	The method for generating the initial guess of the spectrum for the selected component. "Auto" means this is automatically determined by the software. Alternatively, you can used a loaded spectrum as the initial guess.
Lock	The lock option determines whether the spectrum is allowed to change. If this option is chosen, the spectrum of that component is not updated during unmixing.
Unimod	Choose this option to apply the unimodality constraint. Unimodality forces the spectrum to have only one peak (one extremum). however, small magnitude extrema are allowed if they are less than the Unimod Tolerance value. This tolerance value limits the rising slope of the second spectral peak. For example, 5% tolerance means that the increase in magnitude of the neighboring nodes cannot exceed 5%.
HP	Sets a high pass filter for the spectrum. Signal below the HP cut-off frequency is forced to zero. Choose N/A to turn off the high pass filter. Otherwise, the value represents the high pass cut-off frequency. This constraint can help isolate components that are physically mixed and difficult to distinguish.
LP	Sets a low pass filter for the spectrum Signal above the LP cut-off frequency is forced to zero. Choose N/A to turn off the low pass filter. Otherwise, the value represents the cut-off frequency of the low pass cut-off frequency. This constraint can help isolate components that are physically mixed and difficult to distinguish.
Sort	Choose this option to automatically sort the unmixed spectra in ascending order of their center wavelength.
Force	Choose this option to force the first component to non-zero everywhere.
Denoise by PCA	Determines how much of the data will be filtered by principal component analysis. Stronger denoising means less principal components will be used in the data and more details will be lost. Stronger denoising also may slow down the unmixing.
Normalization	Determines how to normalize the unmixed spectra. Equal Height normalizes by the maximum of the spectra. Equal Length normalizes by the sum of the spectra.
Unimod Tolerance (%)	The threshold for the unimodality constraint. It is the percentage of overshoot allowed for the second spectral peak.
PCA	
Mode	PCA can be performed on the original data, the correlation matrix of the original data, or the covariance matrix of the original data.
	Click to display the explained variance.
	Click to display the biplot graph.

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10 3D Reconstruction of Sources

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The Living Image® software includes algorithms for 3D reconstruction of bioluminescent or fluorescent sources (*tomographic analysis*):

- Diffuse Tomography (DLIT™) algorithm - For tomographic analysis of bioluminescent sources. Analyzes a bioluminescent image sequence obtained on the IVIS® Imaging System 200 or 3D Series.
- Fluorescent Tomography (FLIT™) algorithm - For tomographic analysis of fluorescent sources. Analyzes a fluorescent image sequence obtained on the IVIS Spectrum imaging system.

10.1 3D Reconstruction of Bioluminescent Sources

The steps to perform 3D reconstruction of bioluminescent sources include:

- Acquire an image sequence.

  IVIS Imaging System 200 Series & IVIS Spectrum: Acquire a photographic and a structured light image, and two or more bioluminescent images at different wavelengths (560 to 660 nm)(Table 10.1).

  or

  IVIS Imaging System 3D Series: Acquire photographic, structured light, and luminescent images at multiple angles (0-315 degrees) and multiple wavelengths (Figure 10.1).
- Generate the surface topography (*mesh*) of the subject.
- Specify the user-modifiable DLIT algorithm parameters (for example, analysis wavelengths, source spectrum, and tissue properties).
- Reconstruct the position, geometry, and strength of the luminescent sources.

For more details on the DLIT algorithm, see *3D Reconstruction of Light Sources*, page 189.

General Considerations

Animal Requirements

The best surface topography reconstruction is obtained from nude mice. Furred mice, especially those that are symptomatically stressed, do not reflect the structured light well. DLIT analysis cannot be performed without the reconstructed animal surface that is derived from the structured light images. If the study requires a furred mouse strain, then the fur must be removed from the mouse body before imaging, by shaving or applying a depilatory.

Luminescent Exposure vs. Luciferin Kinetic Profile

It is important to consider the luciferin kinetic profile when you plan the image sequence acquisition. The DLIT algorithm currently assumes a flat luciferin kinetic profile. Therefore, to optimize the signal for DLIT 3D reconstruction, carefully plan the start and finish of image acquisition and ration the exposure time at each emission filter so that the sequence is acquired during the flattest region of the luciferin kinetic profile.

The IVIS® Imaging System 3D Series requires about 45 seconds for the instrument components (filter wheel, stage, and mirror) to rotate into position and acquire a photographic and structured light image at a particular view. For all the views, this adds about six minutes to the total acquisition time. As a result, the luminescent exposure time per view and emission filter is limited with respect to the luciferin kinetic profile.

Acquiring an Image Sequence For DLIT Analysis On the IVIS® Imaging System 200 Series & IVIS Spectrum

Table 10.1 shows the recommended image sequence for DLIT analysis. Analyzing more images usually produces more accurate results. At a minimum, the sequence must include data from at least two different emission filters (560-660 nm):

- Emission filter #1: Photographic, luminescent, and structured light image.
- Emission filter #2: Luminescent image.

Table 10.1 Recommended image sequence for DLIT analysis on the IVIS Imaging System 200 Series & IVIS Spectrum

Image Type	Emission Filter Options					
	560	580	600	620	640	660
Photographic	✓	Select the Reuse Photographs option in the control panel.				
Structured light	✓					
Luminescent	✓	✓	✓	✓	✓	✓

NOTE

The binning level must be the same for all of the luminescent images.

NOTE

For details on how to specify a sequential setup and acquire an image sequence, see Chapter 3, page 17.

Acquiring an Image Sequence For DLIT Analysis On the IVIS® Imaging System 3D Series

The IVIS Imaging System 3D Series has a translational stage and rotating mirror which enables views from multiple angle perspectives around the mouse. For DLIT analysis, the image sequence acquired on the IVIS Imaging System 3D Series must include a:

- Photograph every 30° .
- Photograph and structured light image every 45° .
- :Luminescent image acquired through two different filters every 90° .

shows the recommended image sequence.

NOTE

If an appropriate sequential setup (.xsq) already exists, you can import the sequential setup and edit the exposure times and filters in the sequence Eeor. For more details, see *Working In the Sequence Editor*, page 27.

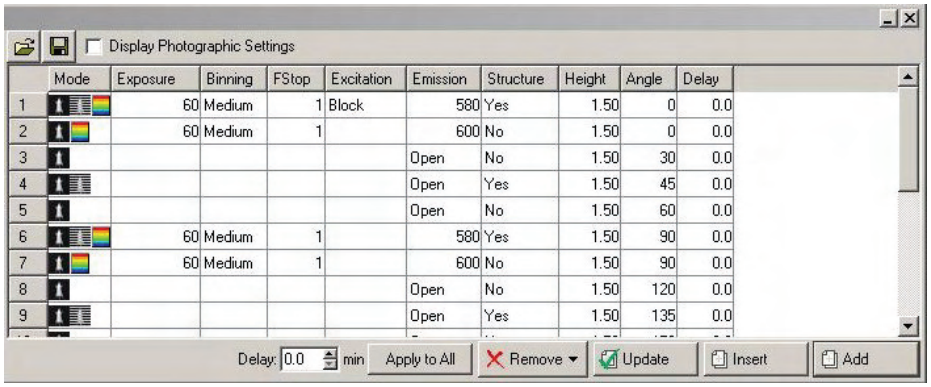

Display Photographic Settings
Mode Exposure Binning FStop Excitation Emission Structure Height Angle Delay
1 60 Medium 1 Block 580 Yes 1.50 0 0.0
2 60 Medium 1 600 No 1.50 0 0.0
3 Open No 1.50 30 0.0
4 Open Yes 1.50 45 0.0
5 Open No 1.50 60 0.0
6 60 Medium 1 580 Yes 1.50 90 0.0
7 60 Medium 1 600 No 1.50 90 0.0
8 Open No 1.50 120 0.0
9 Open Yes 1.50 135 0.0
Delay: 0.0 min
Apply to All
Remove
Update
Insert
Add

Figure 10.1 Sequence editor showing sequence acquisition parameters on the IVIS Imaging System 3D Series for DLIT analysis

NOTE

For details on how to specify a sequential setup and acquire an image sequence, see Chapter 3, page 17.

Reconstructing the Surface Topography (Mesh)

After the image sequence is acquired, reconstructing 3D light sources is a two-step process.

Table 10.2 Steps to reconstruct 3D sources from a bioluminescent image sequence

Step Description	For Details, See	
	IVIS 200 or IVIS Spectrum	IVIS 3D
1. Perform structured light analysis to reconstruct the subject surface topography (mesh).	Figure 10.2, page 112 and Figure 10.3, page 113	Figure 10.2, page 112 and Figure 10.4, page 114
2. Set the DLIT algorithm parameters and reconstruct the position, geometry, and strength of the bioluminescent sources.	Figure 10.5, page 115 and Figure 10.6, page 116	Figure 10.5, page 115 and Figure 10.6, page 116

To reconstruct the surface topography (mesh):

1. Load the image sequence that you want to analyze.
2. In the tool palette, click **Surface Topography**.
   - The Surface Topography and DLIT 3D Reconstruction Tools are displayed.
3. If you want to smooth the surface, confirm the default surface generation options and surface smoothing parameters or enter new values. (For more details, see Table 10.3).
4. If you want to save the results, confirm the default name for the results or enter a new name.
5. Click **Create**.
   - The tomography analysis box appears.

   For the IVIS 200 or IVIS Spectrum, the procedure continues in Figure 10.3.

   For the IVIS 3D, the procedure continues in Figure 10.4.

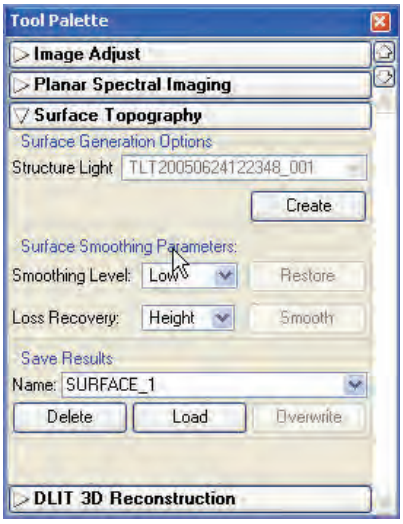
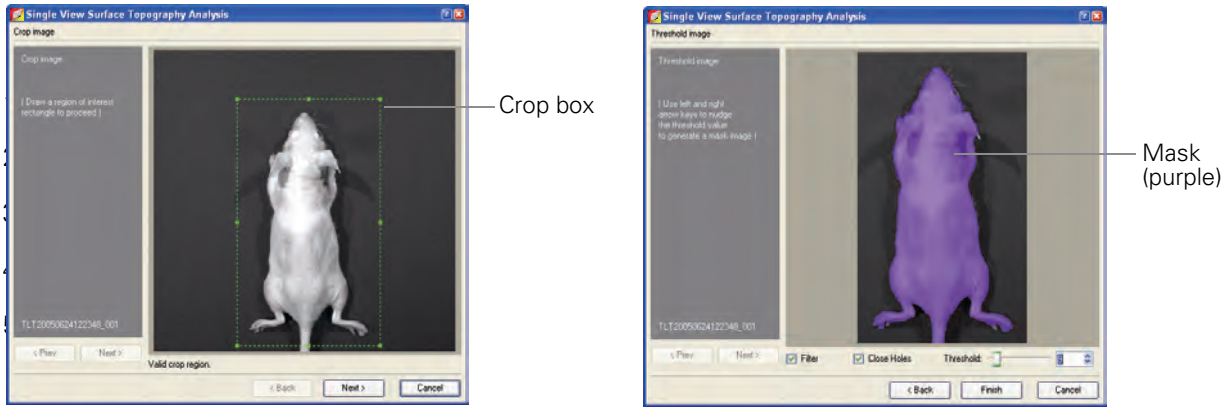


Figure 10.2 Reconstruct the mesh

Table 10.3 Surface topography options & parameters

Item	Description
Surface Generation Options	
Structured Light	A drop-down list of structured light images in the sequence. Select one for use in reconstructing the mesh.
Photo Back Projection	This option is only available for data obtained on an IVIS imaging System 3D Series. If this option is chosen, photographic image data is also used to reconstruct the mesh.
Surface Smoothing Parameters	
Smoothing Level	The amount of smoothing to apply to a reconstructed mesh.
Restore	Removes smoothing that was applied to a mesh.
Loss Recovery	Smoothing can cause loss in the surface volume or height. Make a selection from the drop-down list to reduce losses. 'Height' is recommended for IVIS 200 or IVIS Spectrum surfaces, and 'Volume' is recommended for IVIS 3D surfaces.
Smooth	Initiates the smoothing specified.
Save Result	
Name	The name for the mesh.
Delete	Click to delete the mesh selected from the Name drop-down list.
Load	Click to load the mesh selected from the Name drop-down list.
Save	Click to save the mesh to the name entered in the Name drop-down list.

IVIS® Imaging System 200 Series or Spectrum: Reconstruct the surface topography (mesh) (continued from Figure 10.2):



6. Draw a crop box that includes a one cm margin around the subject, if possible.

7. Click **Next** to display the mask.
   The mask is a purple overlay on the subject image that defines the area of interest for the surface topography reconstruction. The mask should match the underlying photograph of the subject as closely as possible without including any area outside the subject image.

8. If necessary, adjust the threshold value so that the mask fits the subject image as closely as possible without including any area outside of the subject. To change the threshold, do one of the following:
   - Press the left or right arrow keys on the keyboard.
   - Move the Threshold slider left or right.
   - Click the arrows or enter a new value in the box.

9. Click **Finish**.
   - The mesh and 3D tools appear. For more details on the tools, see page 127.

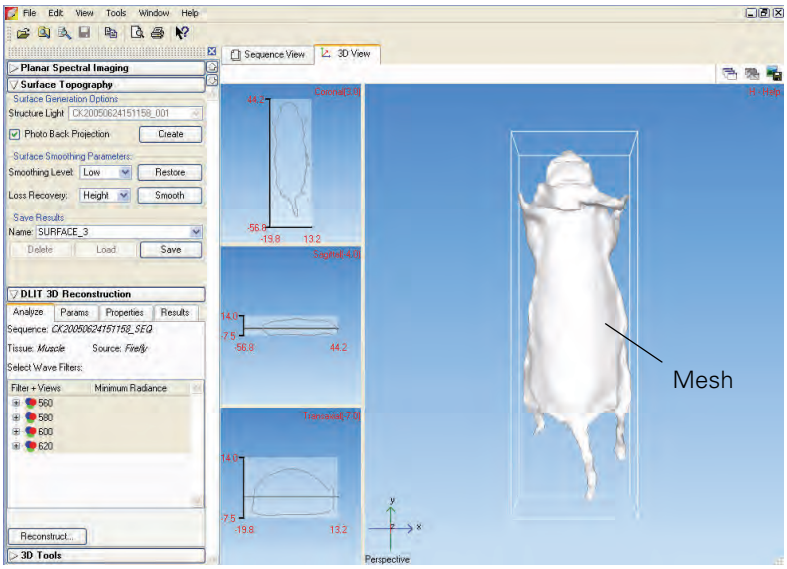
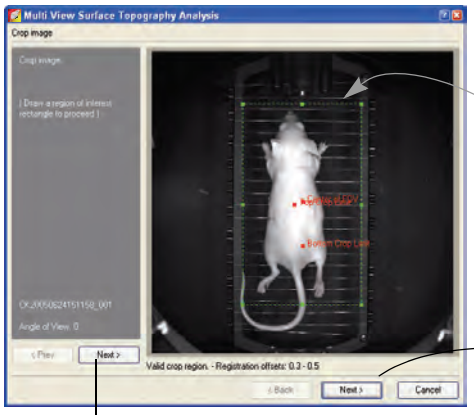


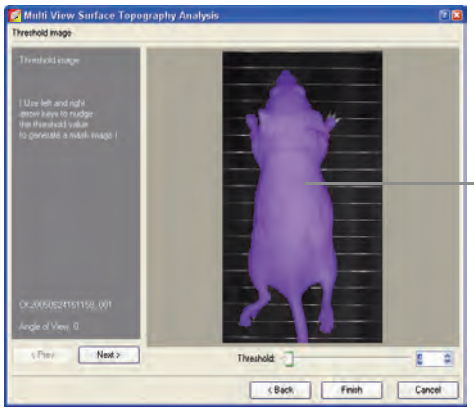
Figure 10.3 IVIS Generating the surface topography, IVIS 200 Series or IVIS Spectrum (continued from Figure 10.2)

IVIS® Imaging System 3D Series: Generating the surface topography (mesh) (continued from Figure 10.2):



6. Confirm that the default crop box includes the subject and a small margin around the subject. If necessary, resize the crop box.

7. Click **Next** to display the next view of the subject. If necessary, resize the crop box to include the subject and a small margin around the subject. Repeat this step to confirm the proper position of the crop box in all views.

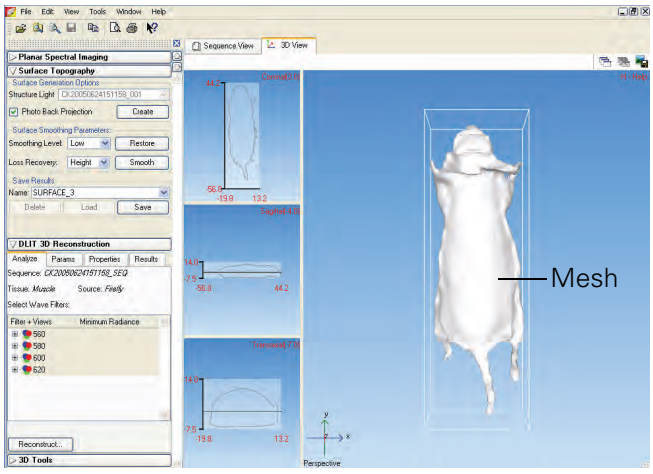


8. Click **Next** to display the mask.
   The mask is a purple overlay on the subject image that defines the area of interest for the surface topography reconstruction. The mask should match the underlying photograph of the subject as closely as possible without including any area outside the subject image.

9. If necessary, adjust the threshold value so that the mask fits the subject image as closely as possible without including any area outside of the subject.

10. Click **Next** to display the next view of the subject. If necessary, adjust the threshold value.

11. Repeat step 10 until the mask is properly adjusted in each view of the image.



12. Click **Finish**.
    - The mesh and 3D tools appear. For more details on the 3D tools, see page 127.

Note: It may take several minutes to generate the mesh. If you selected both the Surface and DLIT options, the software generates the mesh and performs 3D reconstruction of the bioluminescent sources.

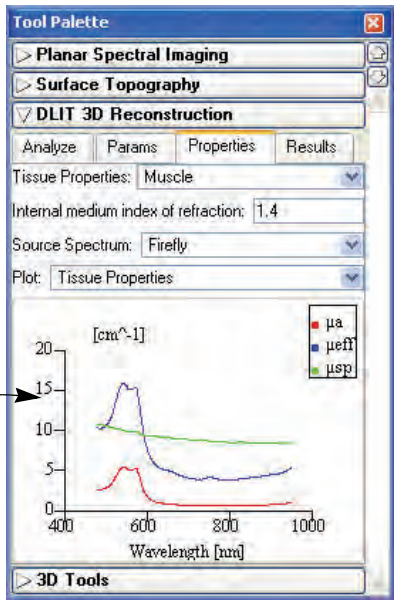
Figure 10.4 Generating the surface topography, IVIS Imaging System 3D Series (continued from Figure 10.2)

Reconstructing 3D Bioluminescent Sources

After the mesh is generated, the 3D reconstruction of the light sources can proceed. Figure 10.6 shows the steps to reconstruct 3D sources and Figure 10.7 shows example results. For more information on the DLIT algorithm and user-modifiable parameters, see Appendix F, page 194.

To reconstruct 3D bioluminescent sources:

1. In the tool palette, click **DLIT 3D Reconstruction**.
2. In the Properties tab, make a selection from the Tissue Properties and Source Spectrum drop-down lists.
   "Muscle" is usually the best choice of tissue properties for general *in vivo* reconstructions.
   Note: The internal medium index of refraction is automatically entered when you select a tissue.
3. If you want to view the tissue properties (μ_a, μ_{eff}, μ'_s) or source spectrum for the tissue and light source selected above, make a selection from the Plot drop-down list in the Properties tab.

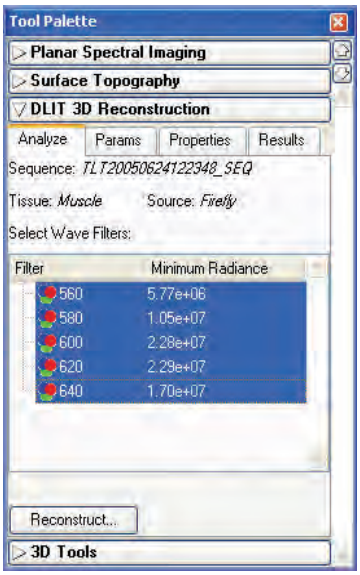


[continued in Figure 10.6]

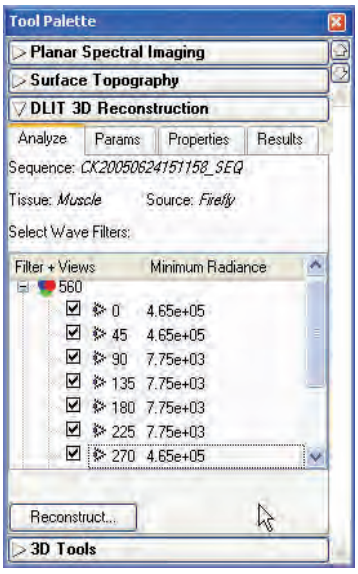
Figure 10.5 Reconstructing 3D bioluminescent sources

To reconstruct 3D bioluminescent sources:
[continued from Figure 10.6]

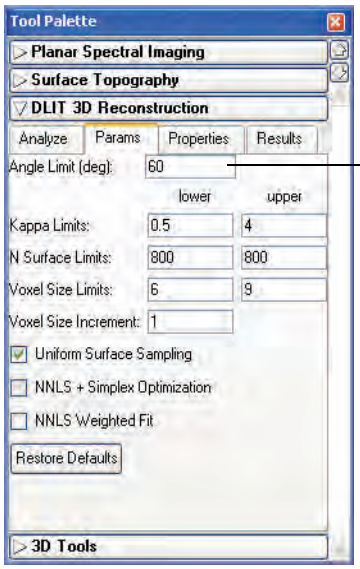
4. In the Analyze tab:
   - IVIS® Imaging System 200 Series or IVIS Spectrum: Select the acquisition wavelengths (560-660 nm).
   - IVIS Imaging System 3D Series: Select the acquisition filters (550-585 nm, 585-620 nm, and 620-700 nm). All view angles are selected by default for each acquisition filter.
5. If necessary, edit the minimum radiance associated with an acquisition wavelength or angle. For more details on the minimum radiance, see Appendix F, page 189.
   Note: It is recommended that you only analyze images that have signal well above the noise.
6. To edit the minimum radiance, double-click the entry and enter a new value.
7. In the Params tab, confirm the parameter defaults or enter new values.
   For more details on the parameters, see Appendix F, page 194.
8. In the Analyze tab, click **Reconstruct**.
   The reconstruction requires about 1-5 minutes, depending on the parameter settings and the processor speed.
   Figure 10.7 shows example 3D reconstruction results.



Analyze tab,
IVIS Imaging System 200 Series or IVIS Spectrum



Analyze tab,
IVIS Imaging System 3D Series



Angle limit default is 60° for IVIS Imaging System 3D Series data or 70° for IVIS Imaging System 200 Series or IVIS Spectrum data.

DLIT algorithm user-modifiable parameters

Params tab

Figure 10.6 Reconstructing 3D luminescent sources

The 3D View in the image window displays the mesh, the 3D reconstruction of the bioluminescent light sources inside the subject (voxels), and the photon density map (photons/mm³ just below the surface).

Results tab displays the results data and the DLIT parameter values.

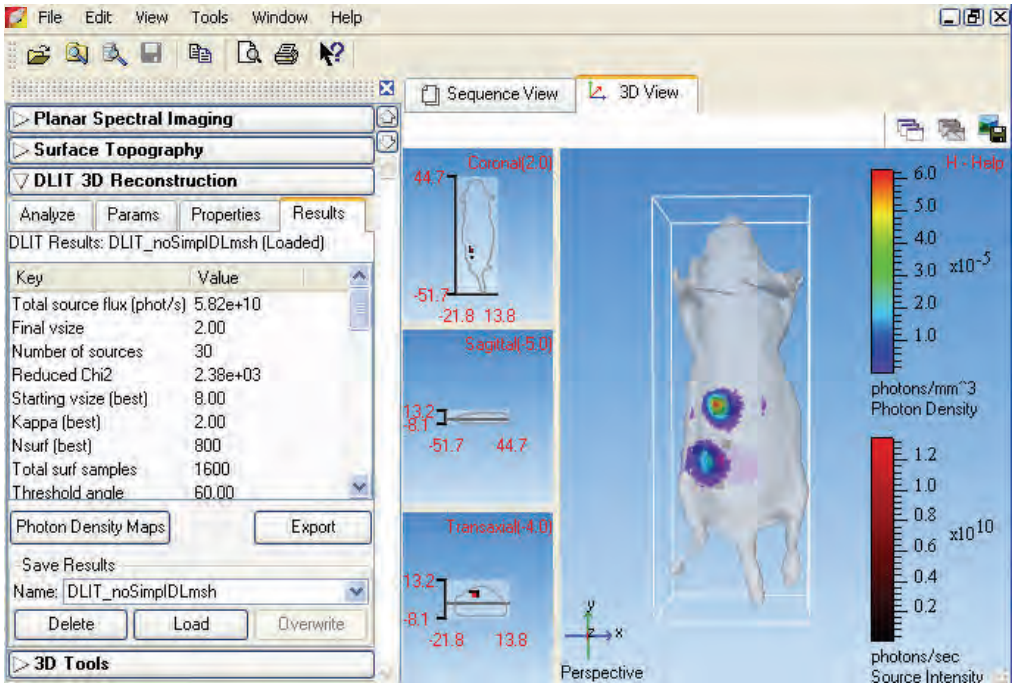


Image window, 3D view

To best view the light sources (voxels):

1. In the 3D tools, move the slider or enter an opacity value to adjust the mesh opacity.
2. Clear the Render Photon Density Map option to display the mesh without the photon density map.

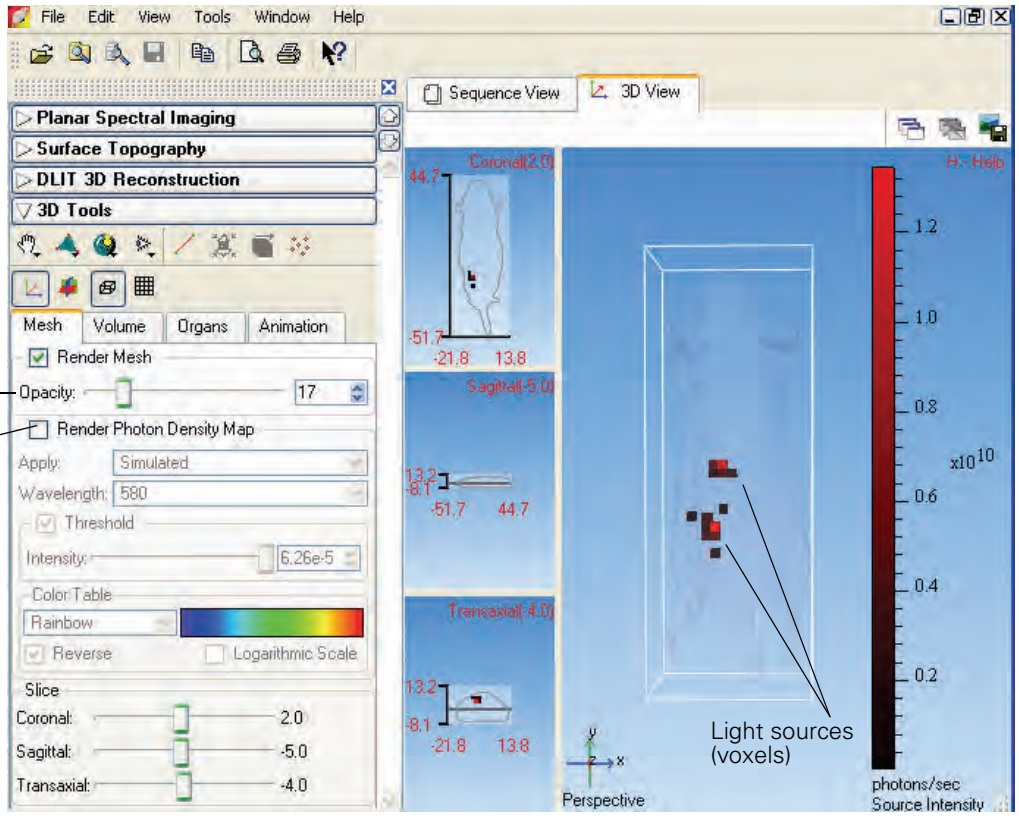


Figure 10.7 Viewing DLIT 3D reconstruction results, Results tab (top) and 3D tools (bottom)

10.2 3D Reconstruction of Fluorescent Sources

The steps to perform 3D reconstruction of fluorescent sources include:

- Acquire a fluorescent image sequence in transillumination mode on the IVIS Spectrum.
- Generate the surface topography (*mesh*) of the subject.
- Specify the user-modifiable FLIT algorithm parameters (for example, transillumination source positions and tissue properties).
- Reconstruct the position, geometry, and strength of the fluorescent sources.

Acquiring an Image Sequence For FLIT Analysis On the IVIS® Spectrum

Table 10.4 shows the recommended image sequence. Acquire the images in transillumination mode using the same excitation and emission filters from at least four source locations.

Table 10.4 Example image sequence for FLIT analysis

Image Type	Source Location	
	First location	Subsequent locations
Photographic	✓	Use previous photo.
Structured light	✓	Use previous photo.
Fluorescent	✓	✓

NOTE

For details on how to specify a sequential setup and acquire an image sequence, see Chapter 3, page 17.

Reconstructing the Surface Topography (Mesh)

After the image sequence is acquired, reconstructing 3D light sources is a two-step process.

Table 10.5 Steps to reconstruct 3D sources from a fluorescent image sequence

Step Description	For Details, See
1. Perform structured light analysis to reconstruct the subject surface topography (mesh).	Figure 10.8, page 119 and Figure 10.9, page 120
2. Set the FLIT algorithm parameters and reconstruct the position, geometry, and strength of the fluorescent sources	Figure 10.6, page 116

To reconstruct the surface topography (mesh):

1. Load the image sequence that you want to analyze.
2. In the tool palette, click **Surface Topography**.
   - The Surface Topography and FLIT 3D Reconstruction tools are displayed.
3. Confirm the default surface generation options and surface smoothing parameters or enter new values. (For more details, see Table 10.6, page 121).
4. Confirm the default name for the results or enter a new name.
5. Click **Create**.
   - The tomography analysis box appears.
6. Draw a crop box that includes a one cm margin around the subject, if possible.

[continued in Figure 10.9]

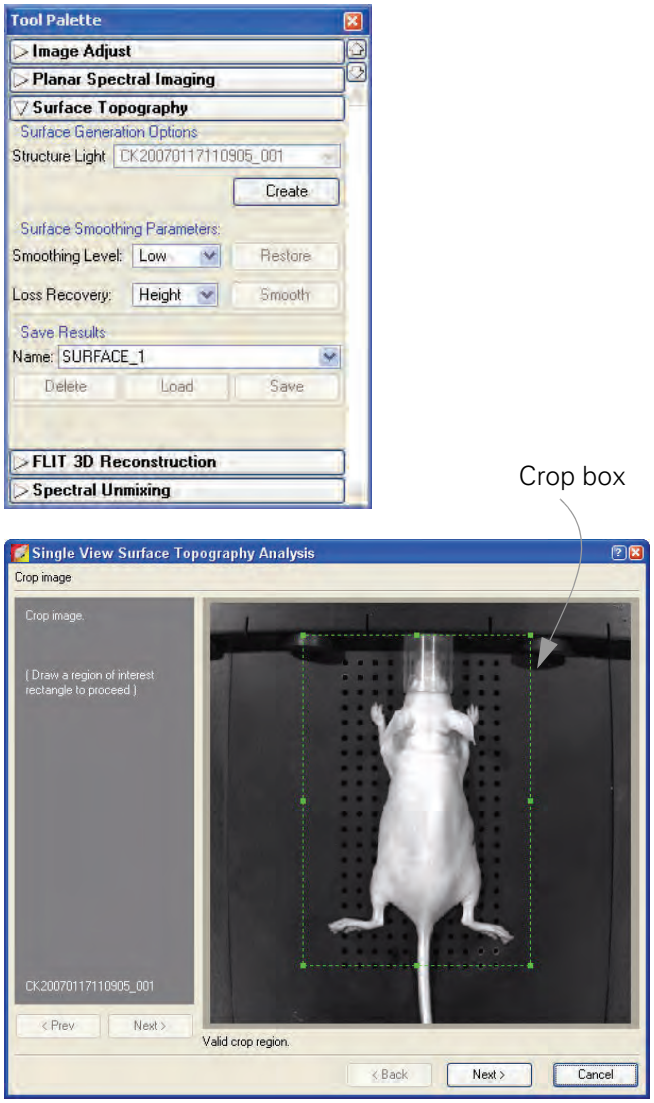
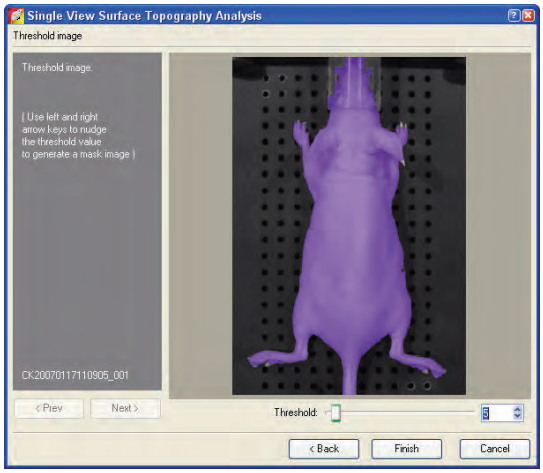


Figure 10.8 Reconstructing the mesh

To reconstruct the surface topography (mesh (continued from Figure 10.8):



7. Click **Next** to display the mask.
   The mask is a purple overlay on the subject image that defines the area of interest for the surface topography reconstruction. The mask should match the underlying photograph of the subject as closely as possible without including any area outside the subject image.

8. If necessary, adjust the threshold value so that the mask fits the subject image as closely as possible without including any area outside of the subject. To change the threshold, do one of the following:
   - Press the left or right arrow keys on the keyboard.
   - Move the Threshold slider left or right.
   - Click the arrows or enter a new value in the box.

9. Click **Finish**.

10. The mesh and 3D tools appear.
    For more details on the tools, see page 127.

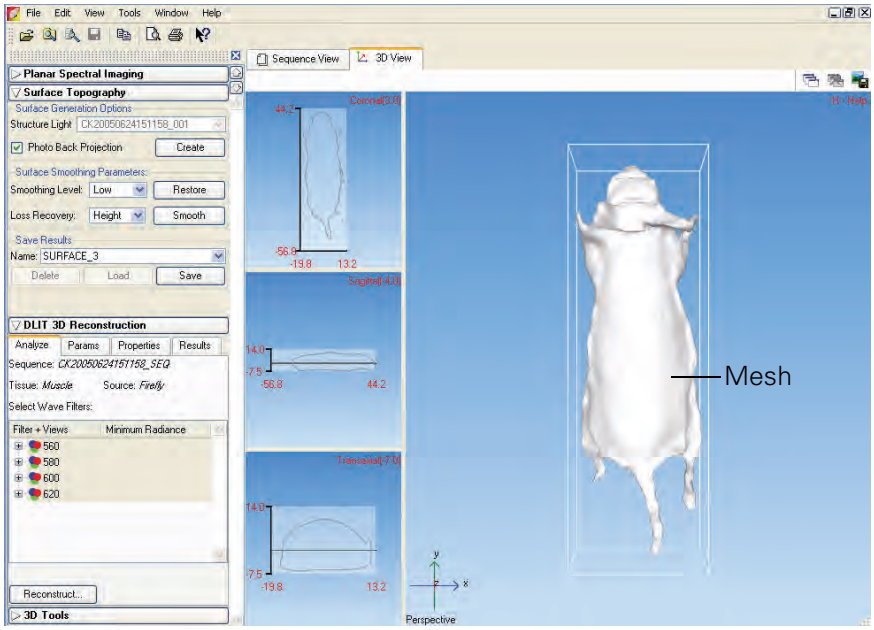


Figure 10.9 Reconstructing the mesh (continued from Figure 10.8)

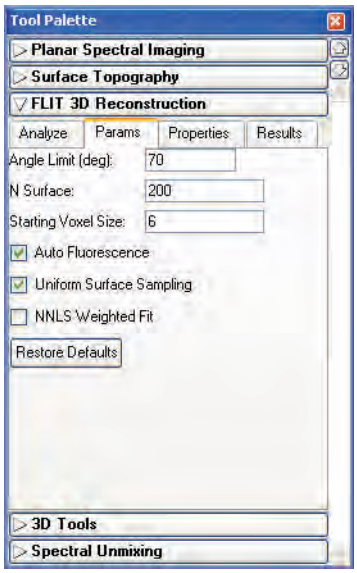
Table 10.6 Surface topography options & parameters

Item	Description
Surface Generation Options	
Structure Light	A drop-down list of structured light images in the sequence. Select one for use in reconstructing the mesh.
Photo Back Projection	This option is only available for data obtained on an IVIS imaging System 3D Series. If this option is chosen, photographic image data is also used to reconstruct the mesh.
Surface Smoothing Parameters	
Smoothing Level	The amount of smoothing to apply to a reconstructed mesh.
Restore	Removes smoothing that was applied to a mesh.
Loss Recovery	Make a selection from the drop-down list (None, Volume, Height).
Smooth	Restores the type of data selected in the Loss Recovery drop-down list (volume, height, or none) that was removed by the smoothing process and applies the selected smoothing level.
Save Result	
Name	The name of the mesh.
Delete	Click to delete the mesh selected from the Name drop-down list.
Load	Click to load the mesh selected from the Name drop-down list.
Save	Click to save the mesh to the name entered in the Name drop-down list.

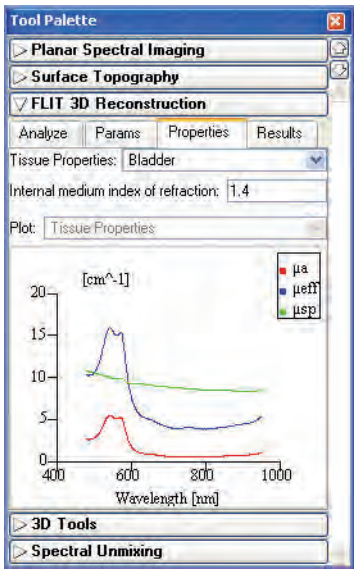
10.3 Reconstructing 3D Fluorescent Sources

After the mesh is generated, the 3D reconstruction of the light sources can proceed. Figure 10.10 shows the steps to reconstruct 3D sources.

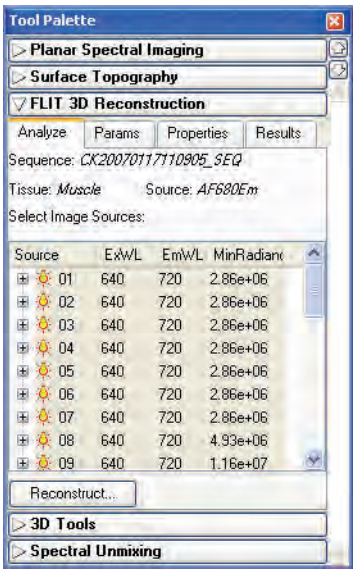
To reconstruct 3D fluorescent sources:



1. In the tool palette, click **FLIT 3D Reconstruction**.
2. In the Params tab, confirm the defaults or enter new values. For more details on the parameters and options, see Appendix F, page 189
3. In the Analyze tab:
   - For images acquired on the IVIS Imaging System 200 Series, select the acquisition wavelengths (560-660 nm).
4. For images acquired on the IVIS® Imaging System 3D Series, select the acquisition filters (550-585 nm, 585-620 nm, and 620-700 nm). All view angles are selected by default for each acquisition filter.



5. In the Properties tab, make a selection from the Tissue Properties and Source Spectrum drop-down lists.
   "Muscle" is usually the best choice of tissue properties for general *in vivo* reconstructions.
   Note: The internal medium index of refraction is automatically entered when you select a tissue.
6. If you want to view the tissue properties (μ_a, μ_{eff}, μ_{sp}) or source spectrum for the tissue and light source selected above, make a selection from the Plot drop-down list in the Properties tab.



7. In the Analyze tab, select the source locations to include in the analysis and click **Reconstruct**.
   Figure 10.7, page 117 shows example 3D reconstruction results.

Figure 10.10 Reconstructing 3D fluorescent sources

10.4 DLIT & FLIT Results

The Results tab displays information about the photon density, voxels, and DLIT algorithm parameters.

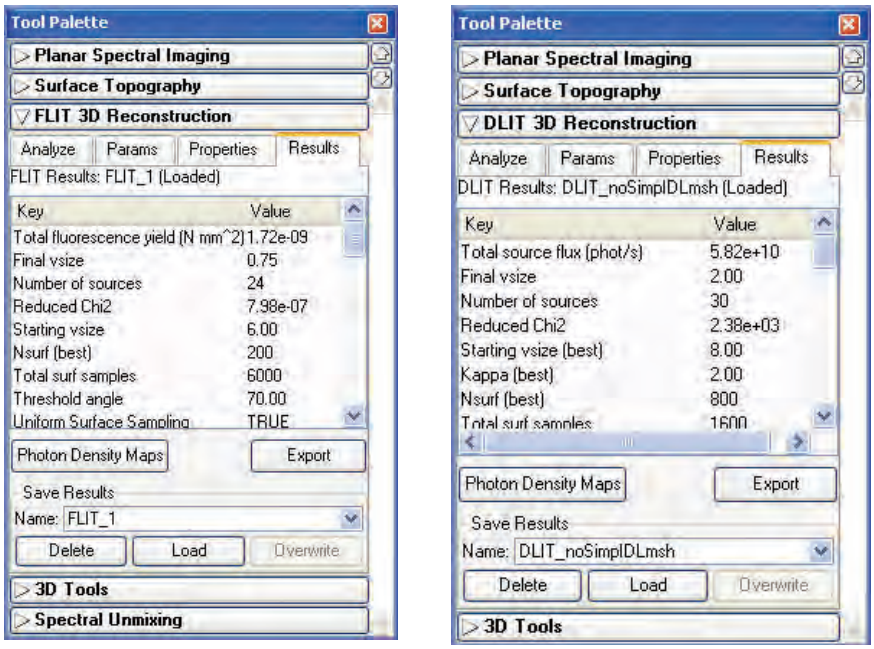


Figure 10.11 3D reconstruction results, FLIT (left) and DLIT (right)

NOTE

For more details on the DLIT and FLIT algorithm parameters, see Appendix F, page 189. Sometimes adjusting the DLIT algorithm parameters improves the fit of the simulated photon density to the measured photon density data.

Item in the Results Tab	Description
Optimized fit parameters	
Total source flux (phot/s) (DLIT result)	The sum of the bioluminescent source intensities.
Total fluorescence yield (N mm^2) (FLIT result)	The total sum of the fluorescent yield. The quantity measured is: (Fluorescence quantum efficiency for the excitation wavelength to emission wavelength photons)*(Excitation wavelength photon absorption cross section)*(Fluorophore number density)*(Volume of voxel size).
Final vsize	The voxel size (length of a side, mm) that produces the optimum solution to the DLIT or FLIT analysis.
Number of sources	The number of voxels that describe the light source(s).
Reduced Chi2	A measure of the difference between the computed and measured photon density maps at the optimum solution. A smaller χ^2 value indicates a better quality of fit.
Starting vsize	The voxel size at the start of the analysis.
Kappa (best) (DLIT result)	The kappa value that produces the optimum solution.

Item in the Results Tab	Description
N surf (best)	The number of surface element data analyzed per wavelengths/images.
Total surf samples	The total number of surface element data analyzed for all wavelengths/images.
Threshold angle	The angle that the object surface normal makes with the optical axis. The optical axis can be considered to be a line perpendicular to the stage. The default setting for this limit is 60° for IVIS® Imaging System 3D Series data or 70° for IVIS Spectrum or IVIS 200 or IVIS Spectrum data. For more details, see *Angle Limit*, page 195.
Kappa limits (DLIT result)	The kappa parameter is a parameter that is searched during a reconstruction to determine the best fit to the image data. For more details, see *Kappa Limits*, page 196.
N surface limits (DLIT result)	The maximum number of surface intensity points to use in the reconstruction for each wavelength. The range is 200 to 800 and the default is 200. The reconstruction time is shorter for smaller values of N (for example, 200). However larger values of N may give a more accurate result because more data are included in the fit.
Starting Voxel Size (FLIT)	The length of the side of the voxel cube in mm units that is evaluated by the algorithm to determine the optimum solution.
Voxel size limits (DLIT result)	The starting voxel size range evaluated by the algorithm to determine the optimum solution.
Voxel size increment (DLIT result)	The incremental change in voxel size evaluated at each iteration during the DLIT analysis.
Uniform Surface Sampling	TRUE = the option is chosen and the surface data for each wavelength is sampled spatially uniformly on the signal area. FALSE = the option is not chosen and the N brighter surface elements are used as data in the reconstruction.
NNLS + Simplex Optimization	TRUE = the option is chosen and a non-negative least squares optimization algorithm is used in addition to the SIMPLEX algorithm to seek the optimum solution. FALSE = only the NNLS algorithm is used to seek the optimum solution.
NNLS Weighted Fit	TRUE = the option is chosen and the DLIT or FLIT algorithm weights the wavelength data inversely proportional to its intensity in the NNLS reconstruction. FALSE = the option is not chosen
Min Radiance	The wavelength image data minimum radiance [photons/sec/cm²/sec] to use in the DLIT or FLIT analysis.
Index of Refraction	The internal medium index of refraction that is associated with the user-selected tissue.
Tissue Properties	The user-specified tissue in which the sources should be located.
Source Spectrum	The emission spectrum of the type of bioluminescent source.
Photon Density Maps	
Simulated	The photon density computed from DLIT source solutions which best fit the measured photon density.
Measured	The photon density determined from the luminescent image measurements of surface radiance.
Wavelength	The wavelength of the photon density map in the active image.
Source Image	The image number of the transillumination source image.
Photon Density Maps	Click to open the Photon Density Maps window.
Save Results	
Name	The default name for the active DLIT or FLIT results.
Delete	Click to delete the selected DLIT or FLIT results.
Load	Click to load the selected DLIT or FLIT results.
Save	Click to save the active DLIT or FLIT results.

Item in the Results Tab	Description
Overwrite	If you reanalyze saved results, click to save the new results and overwrite the previous results.

Viewing Photon Density

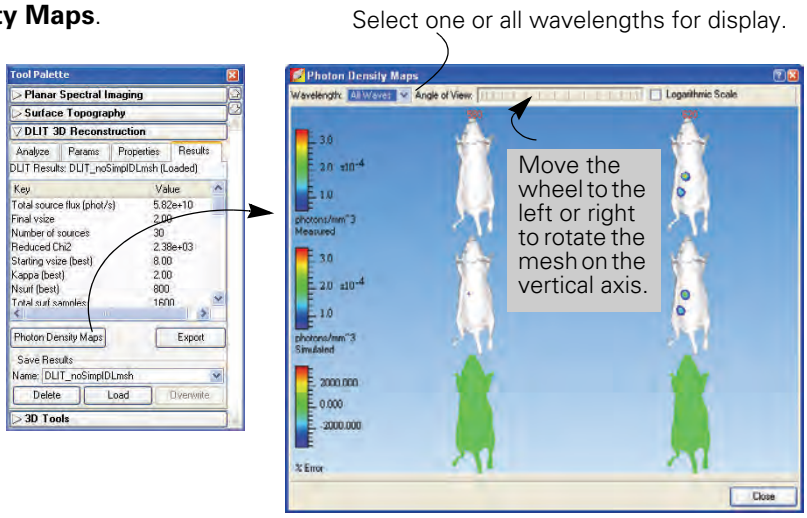
Photon density is the steady state measure of the number of photons in a cubic millimeter. Light sources inside the tissue contribute to photon density in other portions of the tissue. The DLIT or FLIT algorithm first converts the luminescent image of surface radiance to photon density just below the animal surface because this is what can be observed. Then the DLIT or FLIT algorithm solves for point source locations inside the tissue which would produce the observed photon density near the surface.

To check the quality of the DLIT or FLIT construction, it is useful to compare the measured and simulated photon density plots. The photon density is closely related to the measured radiance.

To view the photon density maps:

1. In the Results tab, click **Photon Density Maps**.
   - The Photon Density Maps window displays the photon density maps for all wavelengths.

Note: The voxels are also automatically displayed when the 3D reconstruction is completed. For more details on viewing the voxels, see page 130.



2. To display the measured and simulated photon density profiles:
   a. Select a wavelength.
   b. Drag the crosshairs to the location of interest.
      - The horizontal and vertical photon density profiles are updated.

Note: In a good reconstruction, the measured and simulated photon density curves are close together.

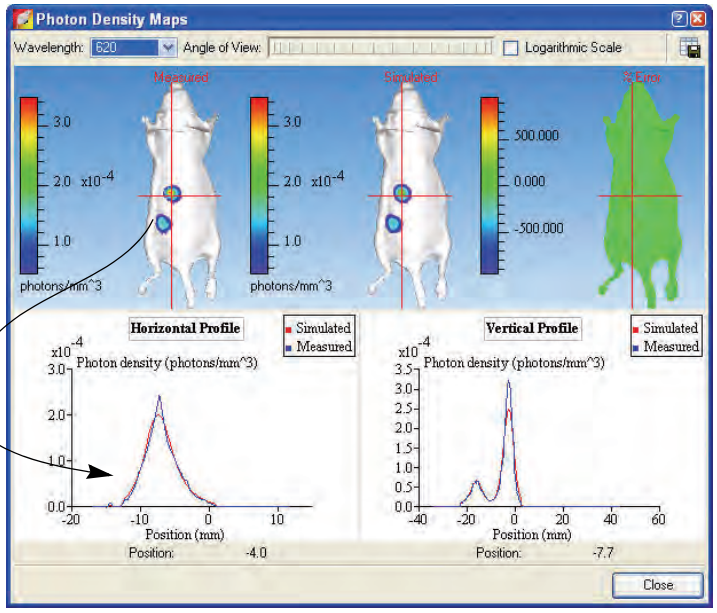


Figure 10.12 How to view photon density maps
Left: Photon density maps for wavelengths. Right: Photon density map at 620 nm.

10.5 Working With 3D Images

The 3D tools appear in the tool palette when a surface topography (mesh) or 3D source is reconstructed, or when you open saved results.

3D Tool Functions:	See Page
View voxel data.	130
Rotate or move the 3D image in the image window.	132
Select a drawing or lighting style for the mesh.	133
Change the view perspective.	135
Display a coronal, sagittal, or transaxial plane through the 3D image and view the resulting cross section (*slice*).	136
Make measurements in the coronal, sagittal, or transaxial section.	136
Display a 3D diagram of an organ(s) on the 3D reconstruction.	137

Click: to select a tool to work with the mesh or 3D results (perspective view only).

to select a mesh drawing style.

to select a shading style for the mesh.

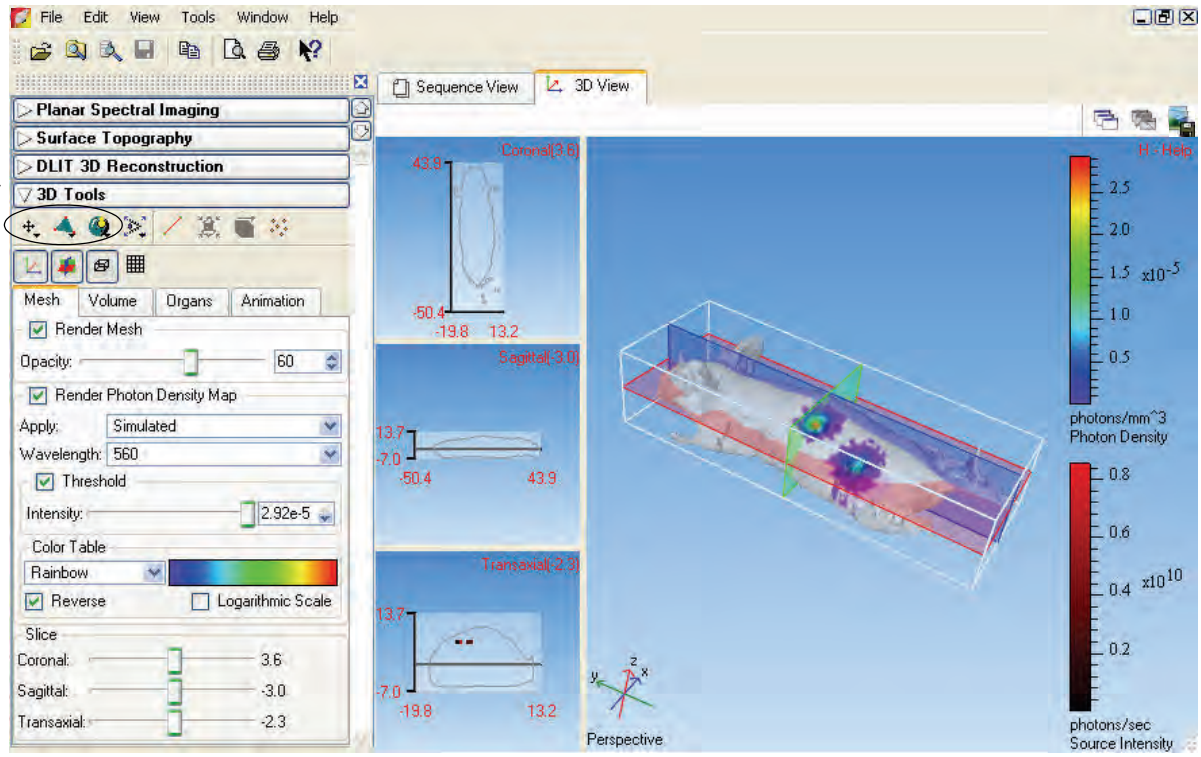


Figure 10.13 3D tools and DLIT results in the 3D view window

3D Tools	Description
Image Tools	A drop-down list of tools for viewing and working with the mesh or DLIT results. (For more details, page 132.) Select to: • Click and display measurement dimensions in the coronal, sagittal, or transaxial view (in the 3D view window). • Drag a measurement cursor in the coronal, sagittal, or transaxial view and display measurement dimensions. (For details on measurement cursors, see page 136.) Select to zoom in or out on the image (use a click-and-drag operation). Select to move the subject in the window (use a click-and-drag operation). Select to rotate the subject around the x, y, or z axis (use a click-and-drag operation).
Drawing Styles	A drop-down list of drawing styles for the mesh (for examples, see Figure 10.13, page 127). The Surface face drawing style ( ) is the default. Point cloud Wire frame Surface face Wire frame and surface face
Shading Styles	A drop-down list of shading styles for the mesh (for examples, see , page 133). The Reflect smooth surface face shading style ( ) is the default. Smooth face Smooth surface face Reflect surface face Reflect smooth surface face
	Select this tool from the drop-down list to change the view perspective (top, bottom, left, right, front, back, or perspective view). For examples of the views, see Figure 10.20.
	Select this tool from the drop-down list to display the perspective view.
	Click to show or hide measurement cursors in the coronal, sagittal, or transaxial views.
	Click to display the manual transform tool.
	Automatic atlas registration tool.
	Click a voxel in the 3D reconstruction, then click this button to display measurements for the voxel in the 3D tools (source voxel measurements).
	Click to hide or show the x,y,z-axis display in the 3D view window.
	Click to hide or show coronal, sagittal, and transaxial planes through the subject in the 3D view window (Figure 10.21).
	Click to show or hide a bounding box around the subject.

3D Tools	Description
	Click to show or hide a grid under the subject.

Mesh Tab	
Mesh	Choose this option to display the mesh in the 3D view window. It may be helpful to turn off the mesh to better view the photon source 3D reconstruction.
Opacity	Controls the mesh opacity. A lower number makes the mesh more transparent. This may be helpful for viewing the photon source 3D reconstruction.
Photon Density Maps	Choose this option to display the photon density map. (If the DLIT reconstruction of the bioluminescent source has not been generated, this option is not available.)
Apply	Select the simulated (computed by the DLIT algorithm) or measured photon density for the photon density map.
Wavelength	Drop-down list of acquisition wavelengths for the active image data. Select an acquisition wavelength for
Threshold	Choose this option to apply a photon density threshold (photons/mm^3) to the photon density map.
Intensity	Use the Intensity slider, the arrows, or enter a value in the box to set the minimum intensity threshold.
Color Table	Specifies the color table for the source intensity scale.
Reverse	Choose this option to reverse the color table. For example, the BlackRed color table represents the source intensity (photons/sec) from low to high using a color scale from black to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to black.
Logarithmic Scale	Choose this option to apply a logarithmic scale to the color table.
Slice	Move the slider to change the position of the coronal, sagittal, or transaxial plane through the mesh. The intersection of the plane and subject (*slice*) is shown in the coronal, sagittal, and transaxial views in the 3D view window (Figure 10.21).
Volume Tab	
Volume	Choose this option to display the voxels.
Min	The minimum voxel intensity (photons/sec).
Max	The maximum voxel intensity (photons/sec).
Render voxels as	A drop-down list of shapes for voxel display.
Threshold	Choose this option to apply a minimum threshold intensity to the voxel display.
Intensity	Use the Intensity slider, the arrows, or enter a value in the box to set the minimum threshold intensity.
Color Table	Specifies the color table for the voxel intensity scale.
Reverse	Choose this option to reverse the color table. For example, the BlackRed color table represents the source intensity (photons/sec) from low to high using a color scale from black to red. If **Reverse** is chosen, the source intensity (photons/sec) from low to high is represented using the color scale from red to black.
Logarithmic Scale	Choose this option to apply a logarithmic scale to the color table.
Source Voxel Measurement	Click the button, then click a voxel in the 3D reconstruction to display measurements for the voxel.
Total Flux	The total photons/sec measured for the voxels selected using the voxel tool.
Volume	Volume of the selected voxels.

3D Tools	Description
Center of mass	The weighted average x, y, and z-coordinates of the selected voxels, where the weights are the flux of each highlighted voxel.
Total Fluorescence Yield	N mm² measured for the voxels selected using the voxel highlight tool.
Organs Tab	
Organs	Choose this option to enable the selection and display of organs on the mesh. (Requires the optional digital mouse atlas.)
Organ database	Choose the male or female organ database from the drop-down list.
Organs	A list of the organs in the selected organ database. Select the organ(s) that you want to display on the mesh.
Reset	Click to display the selected organs in their default positions.
	Click to select all organs in the database and display them on the mesh.
	Click to clear the selected organs and remove all organ diagrams from the mesh.
Update scene	Click to display the selected organs on the mesh.

Viewing Voxel Data

The voxels are automatically displayed when the 3D reconstruction is complete. In the 3D tools, the Volume tab displays the voxel intensity and other data, and provides tools for voxel display.

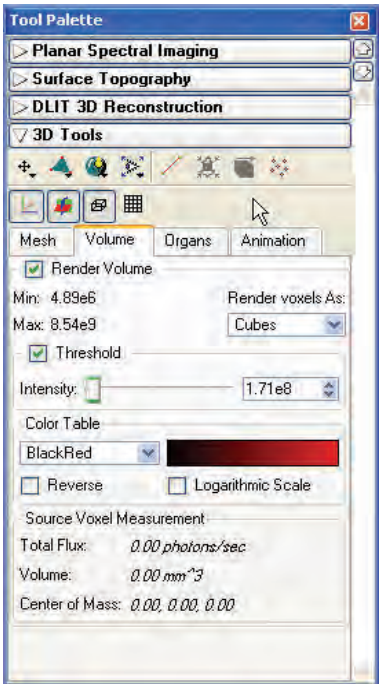
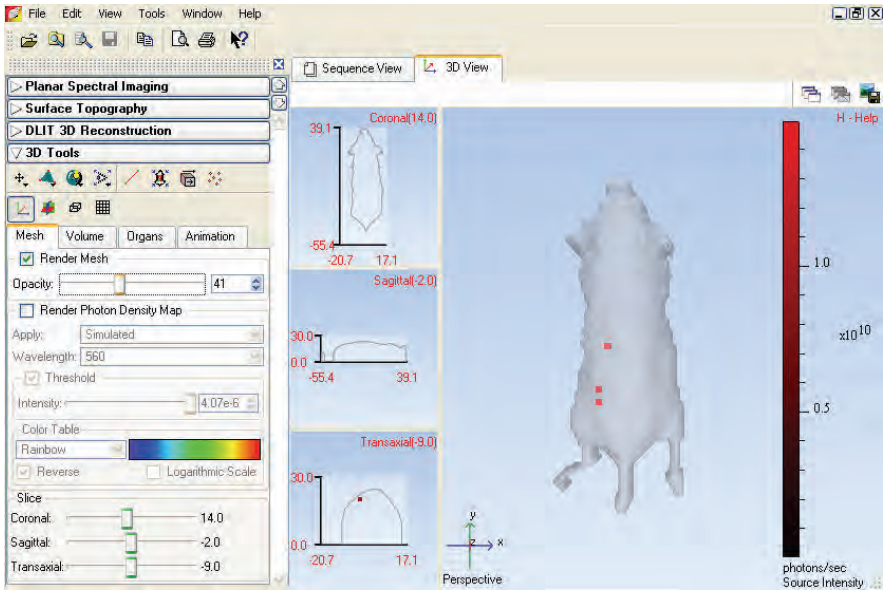


Figure 10.14 3D tools, Volume tab

Figure 10.15 shows how to view the data (total flux, combined volume, and the center of mass) for user-specified voxels. For descriptions of the tools in the Volume tab, see page 129.

To better view the voxel data:

1. In the Mesh tab, reduce the mesh opacity (use the slider or enter an opacity value).
2. Clear the **Render Photon Density Map** option.



To display voxel measurements:

1. Click the **Measure Voxels** button .
2. On the mesh, click the voxel of interest. Alternatively, draw a box around a group of voxels.
   - The Voxel tab displays the selected voxel data.
3. Repeat step 2 to display data for other voxels of interest.
   - The voxel data is updated.
4. To clear the voxel data, click any where in the 3D view window.

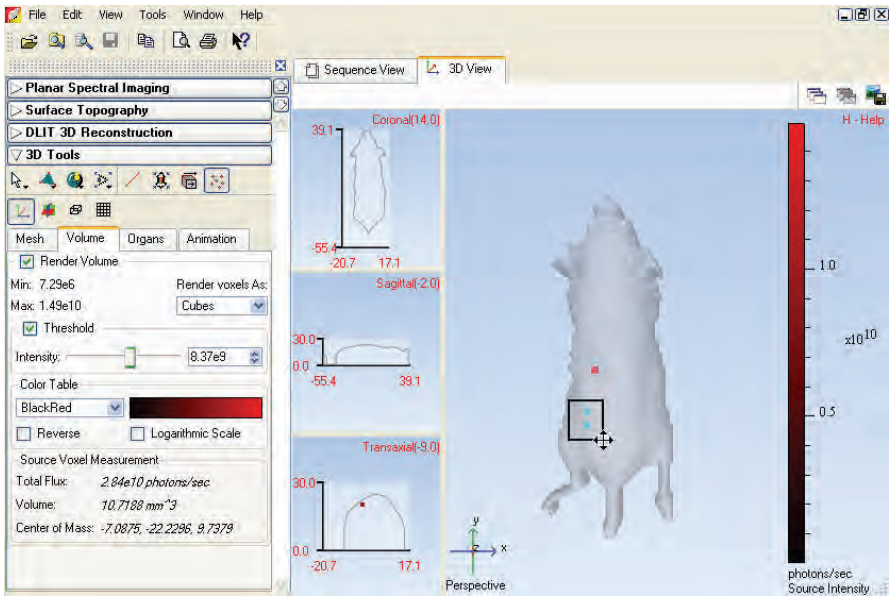
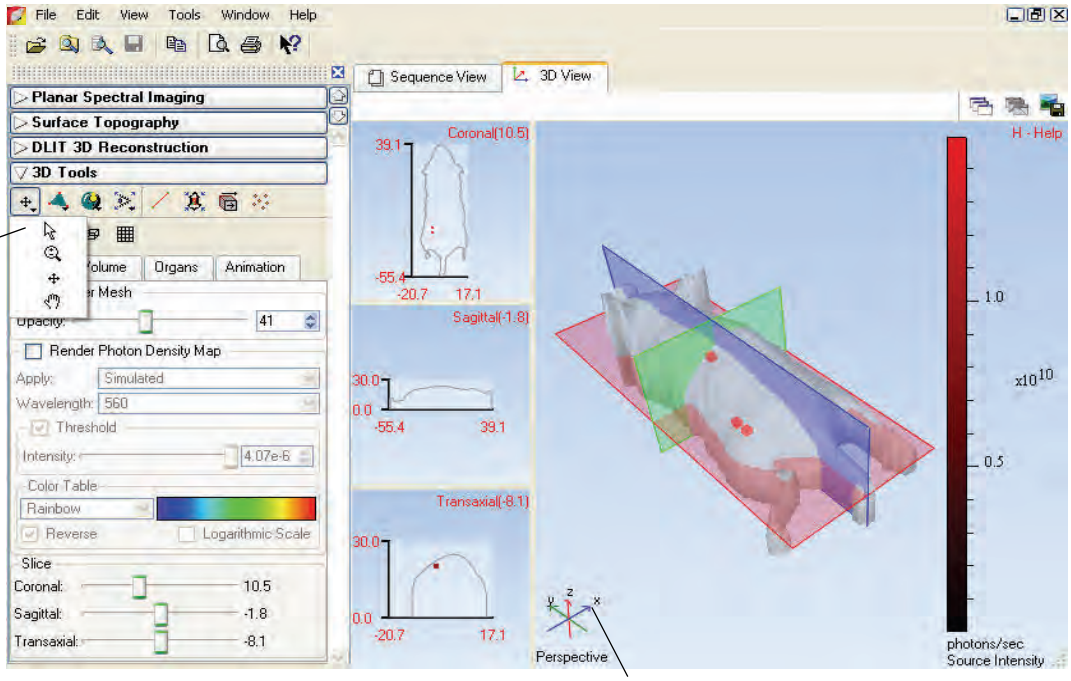


Figure 10.15 Viewing voxel data

Rotating, Moving, or Zooming On a 3D Image



Select a tool from the drop-down list.

Axis shows image orientation.

To rotate the image:

1. Choose the or tool.
2. Place the pointer in the 3D View window.
3. Click and drag the pointer in the x, y, or z-axis direction.
   - The x,y,z-axis shows the orientation of the image

To move the image:

Select the arrow in the 3D tools and drag the image. Alternatively, press the Shift key while you drag the image.

To zoom in or out on the image:

1. Select the arrow in the 3D tools.
2. To zoom in on the image (magnify), right-click (Ctrl+click for Macintosh users) and drag the toward the bottom of the window.
3. To zoom out on the image, right-click and drag the toward the top of the window.

Figure 10.16 Working with images in the 3D View window

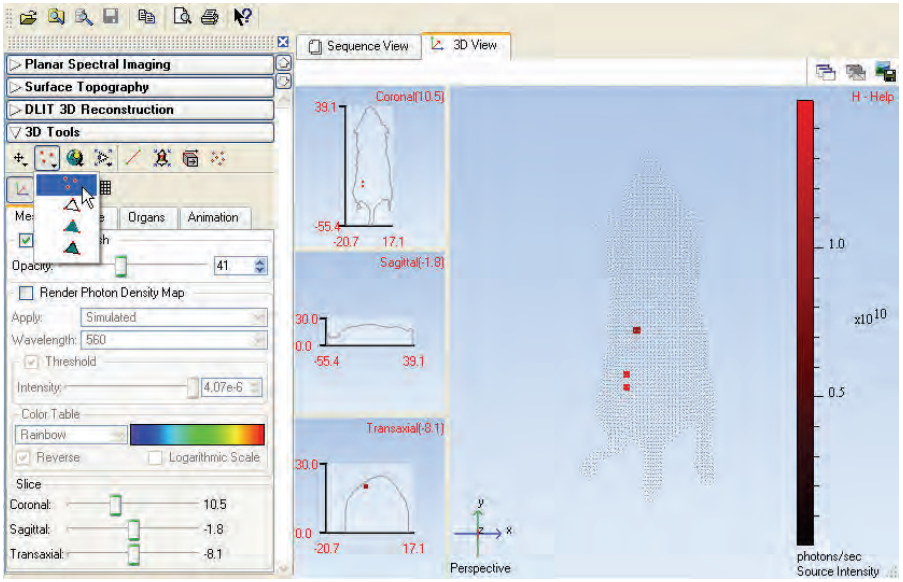
Selecting a Drawing or Lighting Style for the Mesh

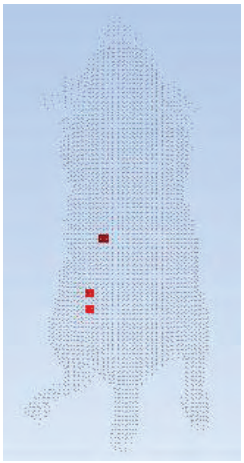
You can choose a different drawing and lighting style to change the appearance of the mesh.

Figure 10.17 shows how to choose a mesh drawing style.

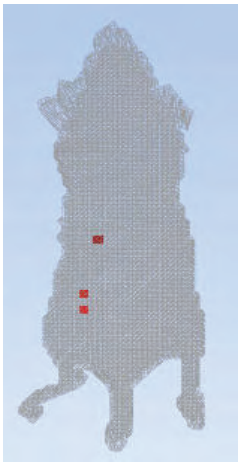
Figure 10.18 shows how to choose a mesh shading style.

To choose a drawing style, make a selection from the Drawing style drop-down list in the 3D tools.

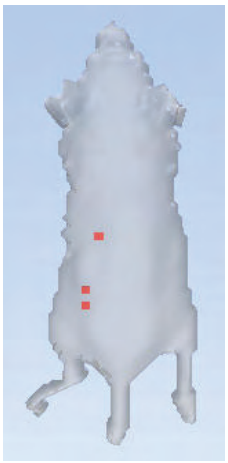




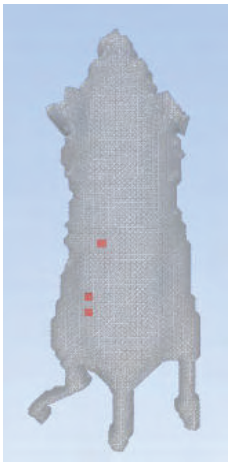
Point cloud mesh



Wire frame mesh



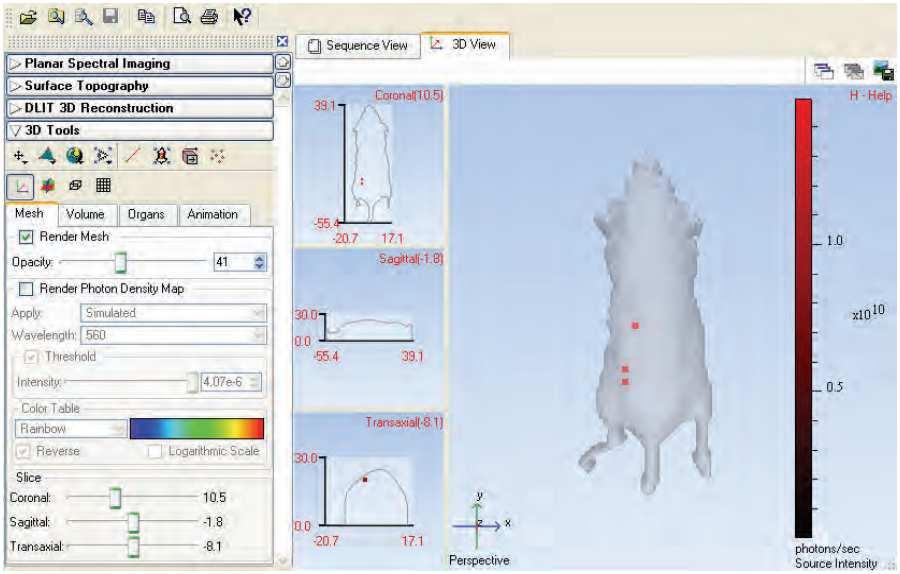
Surface face mesh

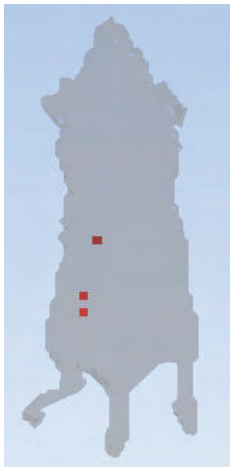


Wire frame & surface face mesh

Figure 10.17 Mesh drawing styles

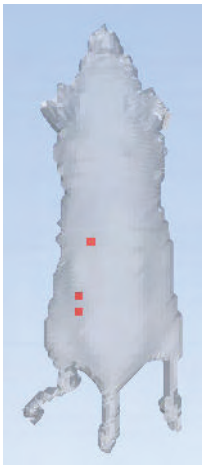
To choose a shading style, make a selection from the Shading style drop-down list in the 3D tools.





Surface face

Smooth surface face



Reflect surface face

Reflect smooth surface face

Figure 10.18 Mesh shading styles

Changing the View Perspective

Figure 10.19 shows how to view a 3D image from different perspectives. Figure 10.20 shows examples of the other available views.

To change the view:

- Select to change the view.
- Alternatively, click the mesh, then press the **V** key to cycle through the different views of the mesh (Figure 10.20).
- Select to display the perspective view.

Note: Only the perspective view can be rotated or moved in the 3D view window.

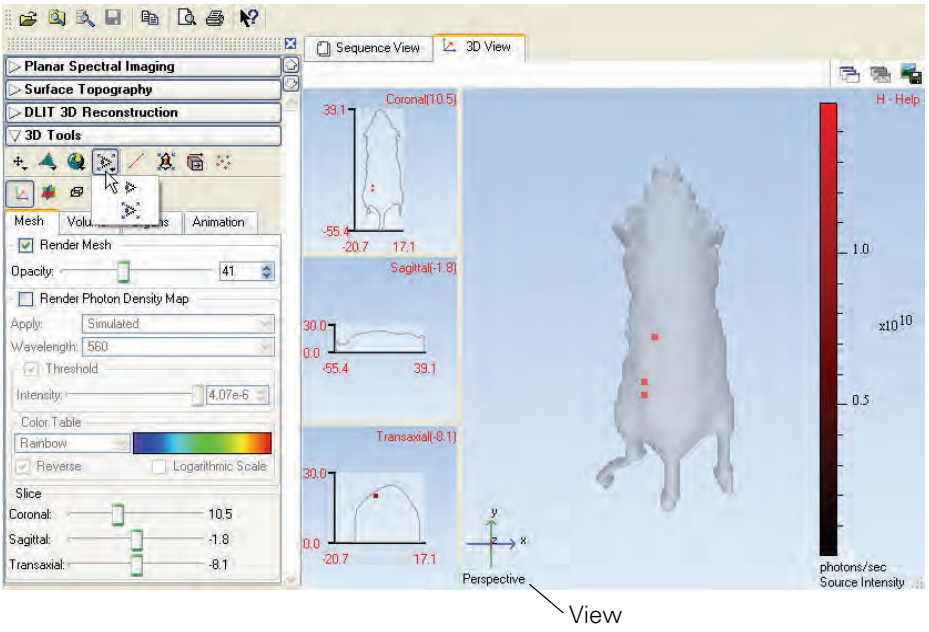
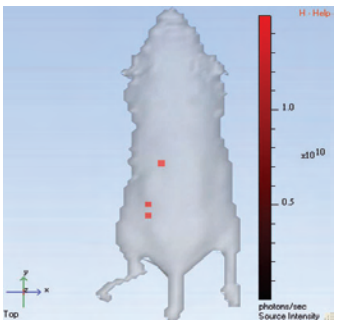
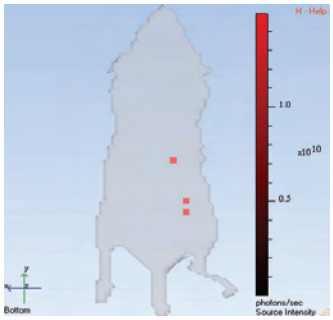
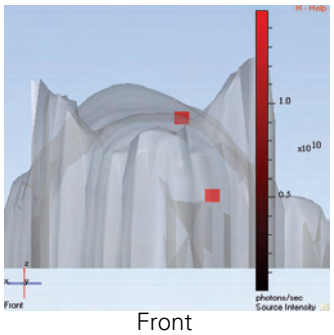
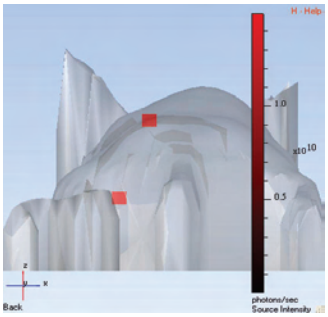


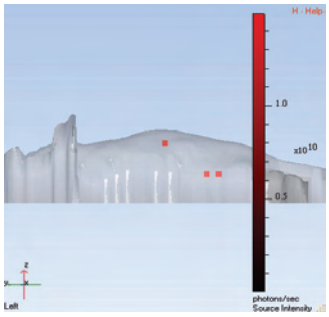
Figure 10.19 Changing the view of the mesh
This view of the mesh, called the perspective view, is the default.


Top


Bottom


Front


Back


Left

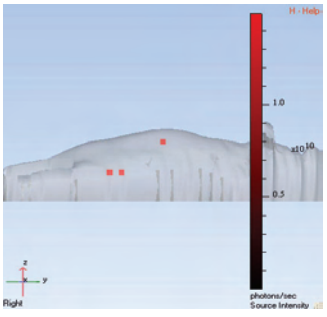

Right

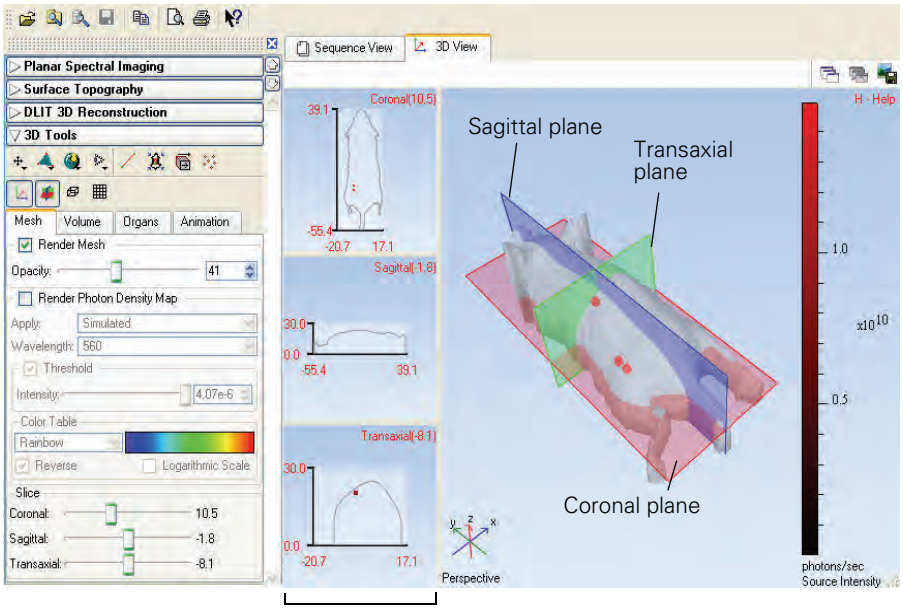
Figure 10.20 Alternative views of the mesh

Displaying Planes Through the 3D Image

Figure 10.21 and Figure 10.22 show how to display planes through the 3D image and make measurements.

To display the planes:

1. Click the button.
   - The Coronal, Sagittal, or Transaxial windowpane shows the intersection of the plane with the image (*slice*).
2. To change the location of a plane, move the coronal, sagittal, or transaxial slider left or right. Alternatively, click the slider, then press the ←or → keyboard arrow keys.
   - The Coronal, Sagittal, or Transaxial windowpane is automatically updated.



Coronal, sagittal, and transaxial windowpanes show the slice through the mesh taken by the associated plane.

Figure 10.21 3D view window and 3D tools

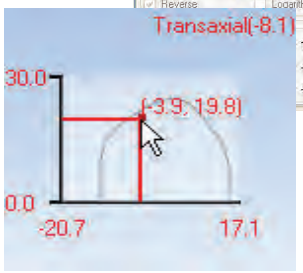
To measure distance in a plane through the subject:

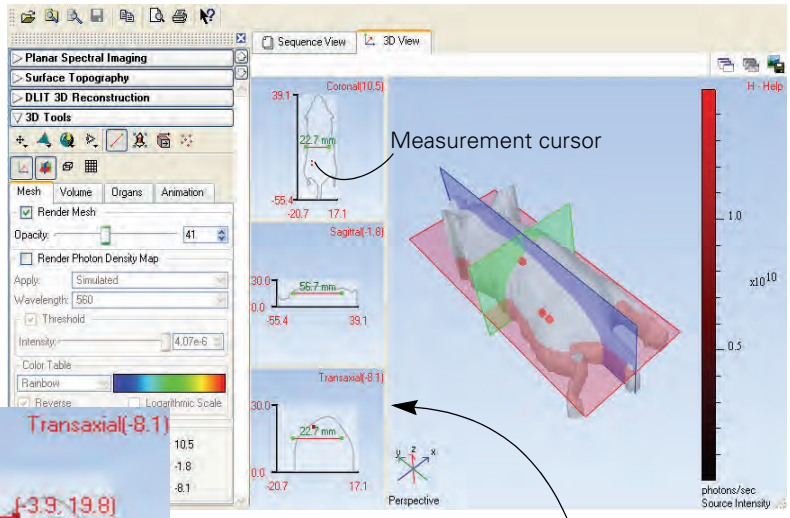
1. Click the button.
   - Measurement cursors and cursor length (mm) are displayed in the Coronal, Sagittal, and Transaxial windowpanes.
2. To measure a distance, drag each end of the cursor to the position of interest.
   - The distance measurement is updated.

To display x,y coordinates:

1. In the Coronal, Sagittal, or Transaxial windowpane, click the position of interest.
   - The x,y-coordinates (mm) of the position are displayed.

   If you drag the cursor, the coordinates are updated.





To change the windowpane size, position the mouse pointer over the vertical or horizontal border. When the ↔ or ↕ appears, click and drag the border.

Figure 10.22 Making measurements and displaying point coordinates in the coronal, sagittal, and transaxial plane

10.6 Displaying Organs on the 3D Reconstruction

The Xenogen digital mouse atlas enables you to display 3D mouse organs on the 3D reconstruction. Figure 10.23 shows how to display organs on the mesh.

The software automatically aligns the organs on the mesh. However, you can also manually adjust the scale or location of organs on the mesh (Figure 10.24 and Figure 10.25).

You can also import a custom organ atlas created from Open Inventor files (.iv).

To display organs on the mesh:

1. In the 3D Tools, click the Organs tab.
2. Confirm that the mesh is in the perspective view (click the button or press the **R** key).
3. Choose the Render Organs option.
4. Select an organ atlas.
   - All of the organs in the selected atlas appear on the mesh.
5. To co-register the digital organs and the mesh:
   a. Click the **Fit organs to the mouse** button .
   b. On the drop-down list that appears, click a button to choose an option:
   Rigid registration - Performs linear transformation, but keeps the shape of the atlas mesh.
   Full registration - Performs linear transformation and volume deformation.
   - The organs are displayed in the anatomically relevant position on the mesh.

Note: For an optimum fit when there is a large difference between the orientation or size of the atlas organs and mesh, first use the transformation tool to manually register the mesh and atlas organs, then click the or tool to automatically fit the organs. (For more details on manual registration, see Figure 10.24.)

6. If necessary, adjust the opacity of the organs using the slider or enter a number in the box.

Note: The organs in the mesh are easier to view if you do not select Skin in the Organs list.

7. To clear all organs from the mesh, click the **Deselect All** button . To remove a specific organ, remove the check mark next to the organ name.
8. To display a specific organ(s), choose the organ name. To display all organs on the mesh, click the **Select All** button .

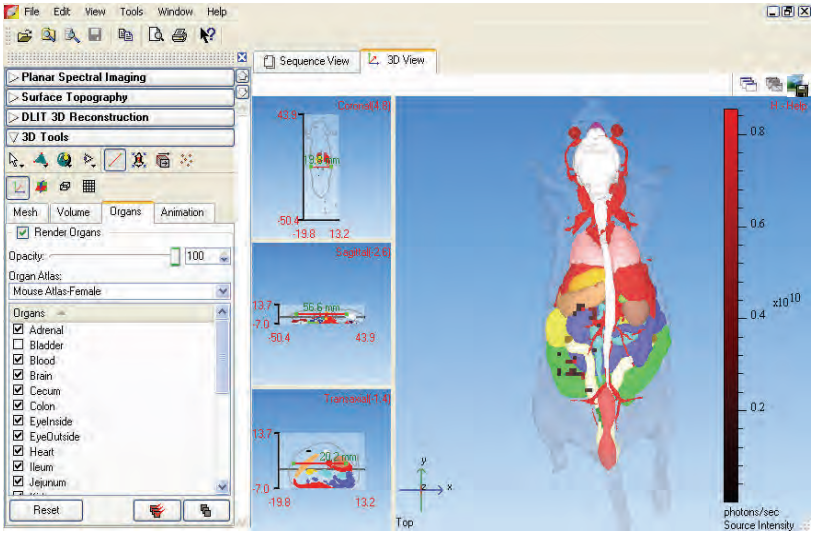
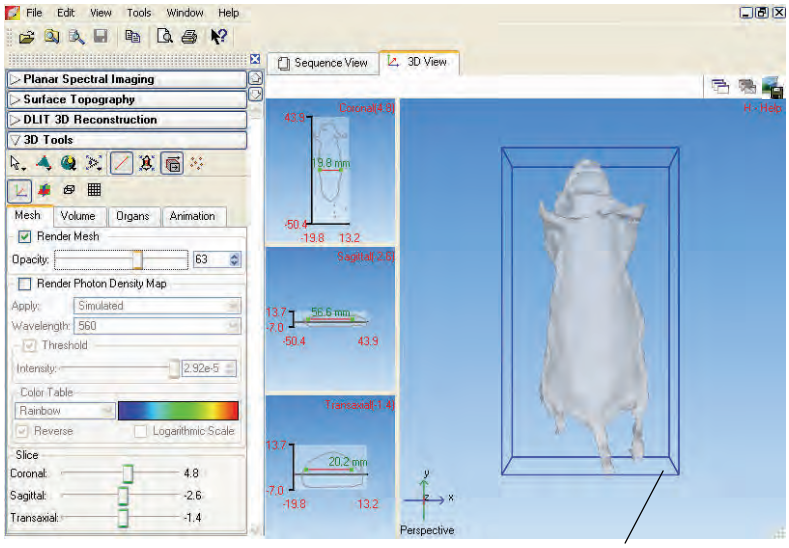


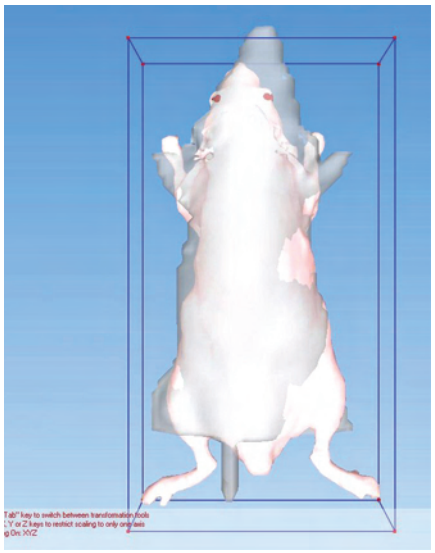
Figure 10.23 Displaying organs on the mesh

To manually adjust the scale or location of organs:

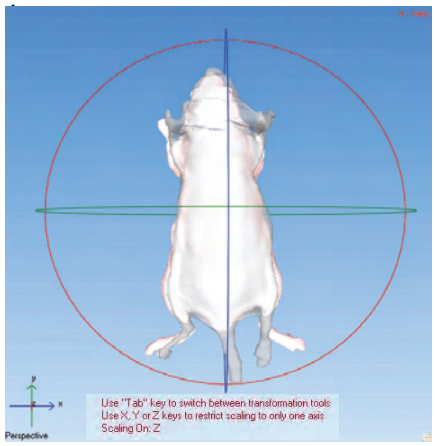
1. Follow step1 to step 4 in Figure 10.23.
2. Click the **Transform tool** button .
   - The transform tool appears.
3. To adjust the x,y, or z-position of the organ, drag the transform tool.
4. To return the transform tool to the default location, click **Reset**.



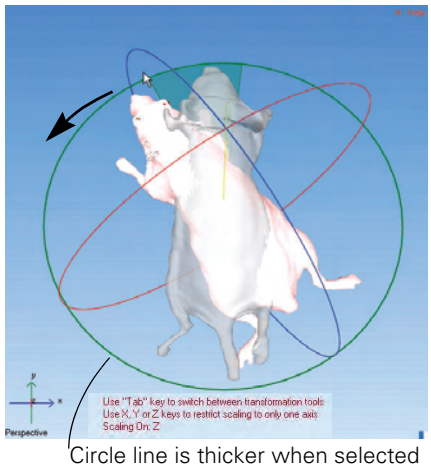
Transform tool



5. Press the **Tab** key to put the transform tool in scale mode.
   - A red cube ■ appears at each corner of the transform tool.
6. To increase or decrease (*scale*) the size of the organ, drag a red cube at a corner of the transform tool. **Note:** To restrict scaling to a particular axis, press the X, Y, or Z key, then drag a red cube.


Use "Tab" key to switch between transformation tools
Use X, Y or Z keys to restrict scaling to only one axis
Scaling On: Z
Perspective

7. Press the **Tab** key again to put the transform tool in rotate mode.
   - A red, green, and blue circle appear around the mesh.


Use "Tab" key to switch between transformation tools
Use X, Y or Z keys to restrict scaling to only one axis
Scaling On: Z
Perspective

Circle line is thicker when selected

8. To rotate the organ on the x,y, or z-axis, click the blue, green, or red circle and drag the mouse arrow in the direction of interest.
   Note: To return the organ drawing to the default position and size, click **Reset** and the button.
9. To turn off the transform tool, click the button.

Figure 10.24 How to manually co-register organs on the mesh

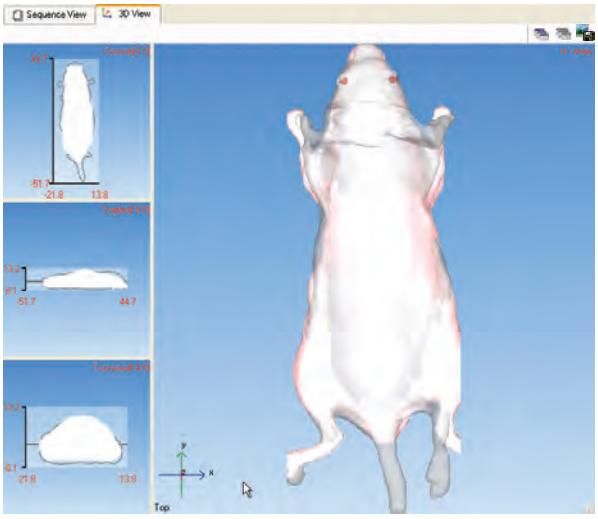
Use the transform tool to move, scale, or rotate the organ. In this example, skin is selected from the organ list. **Note:** To return the organs to the default orientation and scale, click **Reset**.

To check the organ position on the mesh:

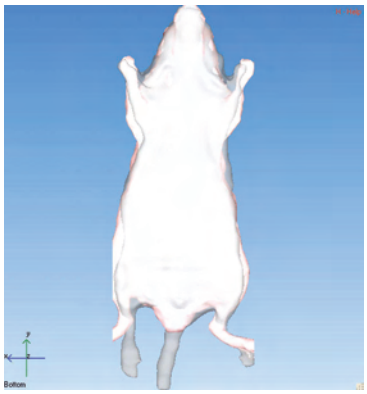
1. Check the organ position in the Coronal, Sagittal, and Transaxial windowpanes.

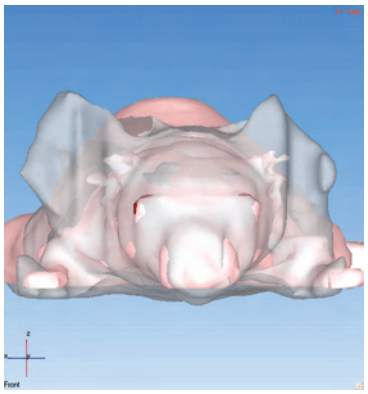
2. In the 3D View tab, click in the windowpane with the mesh.

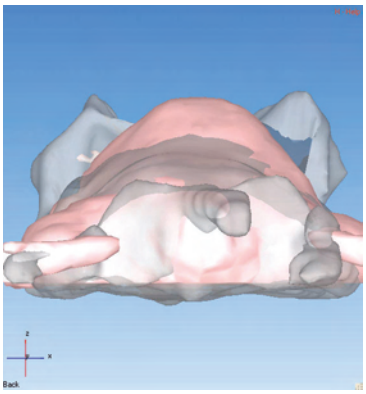
3. Press the **V** key or the button to display alternative views of the mesh.

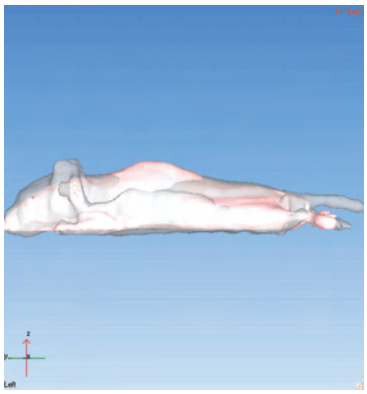


Top


Bottom


Front


Back


Left

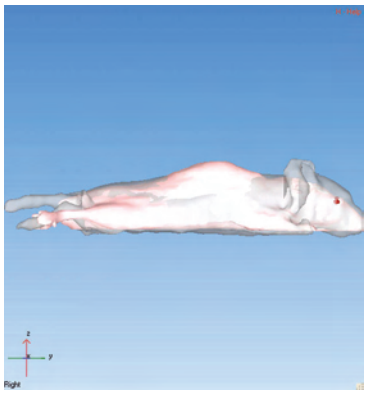

Right

Figure 10.25 Checking organ and mesh alignment
This example shows alternative views of the skin that has been aligned on the mesh.

Importing an Organ Atlas

You can import an organ atlas (Open Inventor format, one organ per .iv) derived from MRI or CT scans.

NOTE

The imported atlas must include a skin file named skin.iv.

To import an organ atlas:

1. Open the DLIT results that are associated with the organ atlas.
2. Select **File →Import →Organ Atlas** on the menu bar.
   - The Import Organ Atlas box appears.
3. Click **Add Organ Files**.
4. In the dialog box that appears, select all of the Open Inventor files that you want to include in the atlas (one .iv per organ) and click **Open**.
5. In the Select Skin Mesh drop-down list, select the skin organ file.
6. Click **Generate Mesh Coefficients**.
7. Enter a name for the atlas and click **Save Organ Atlas**.
   - The organ atlas (.atlas) is created. The atlas name appears in the Organ Atlas drop-down list (in the 3D tools, Organs tab).

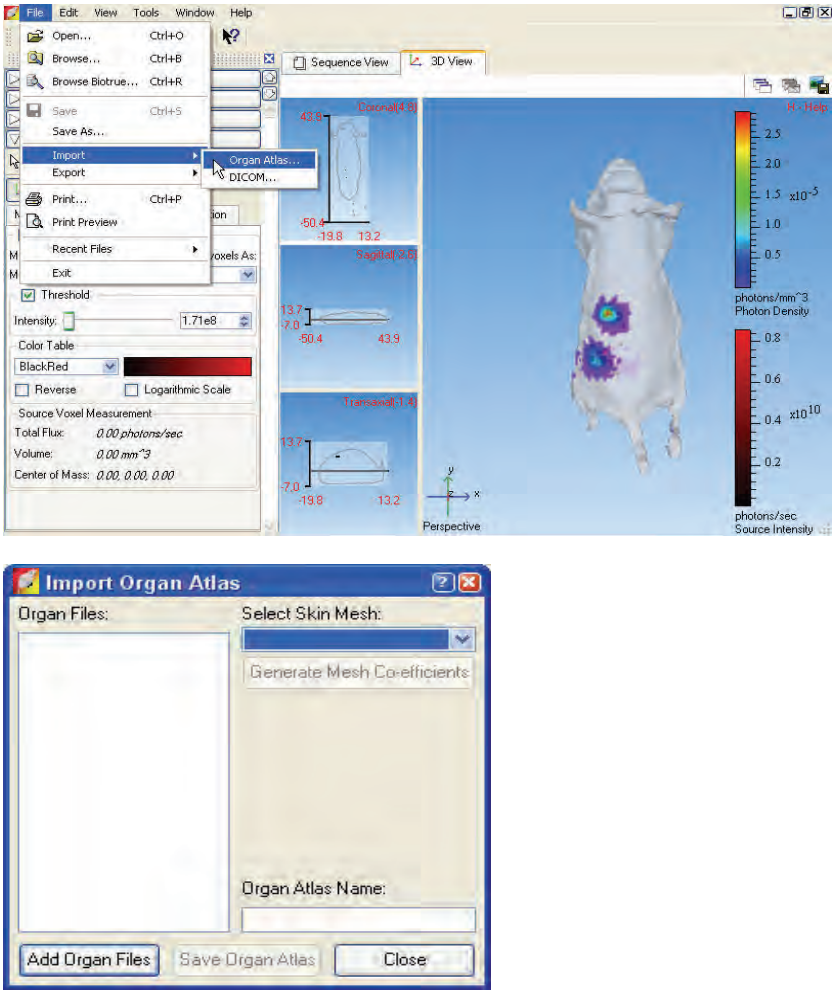


Figure 10.26 Importing an organ atlas

10.7 Managing Results

To save results:

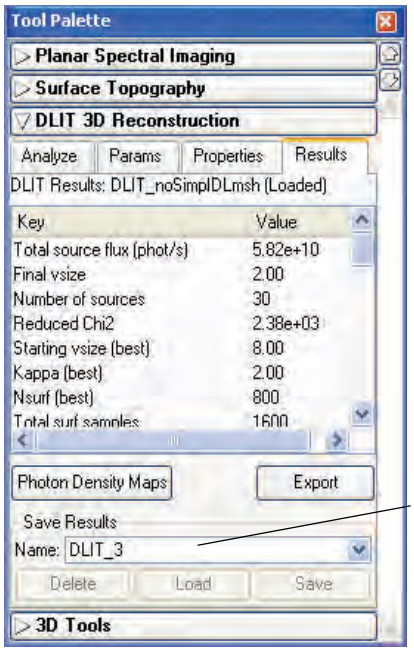
1. In the Results tab of the DLIT/FLIT 3D reconstruction tools, confirm the default file name or enter a new name.
2. Click **Save**.
   - The results are saved to the sequence click number folder and are available in the Name drop-down list.

To open results:

1. In the Results tab, make a selection from the Name drop-down list.
2. Click **Load**.

To copy user-specified results:

1. In the Results tab, select the results of interest.
2. Right-click the results table and select **Copy Selected** from the shortcut menu that appears.



To copy all results:

1. In the Results tab, right-click the results table and select **Copy All** from the shortcut menu that appears.
   - All of the results table is copied to the system clipboard.

To export results:

1. In the results tab, right-click the results table and select Export Results from the shortcut menu that appears.
2. In the dialog box that appears, choose a folder for the results, enter a file name, and click **Save**.

Figure 10.27 Managing results

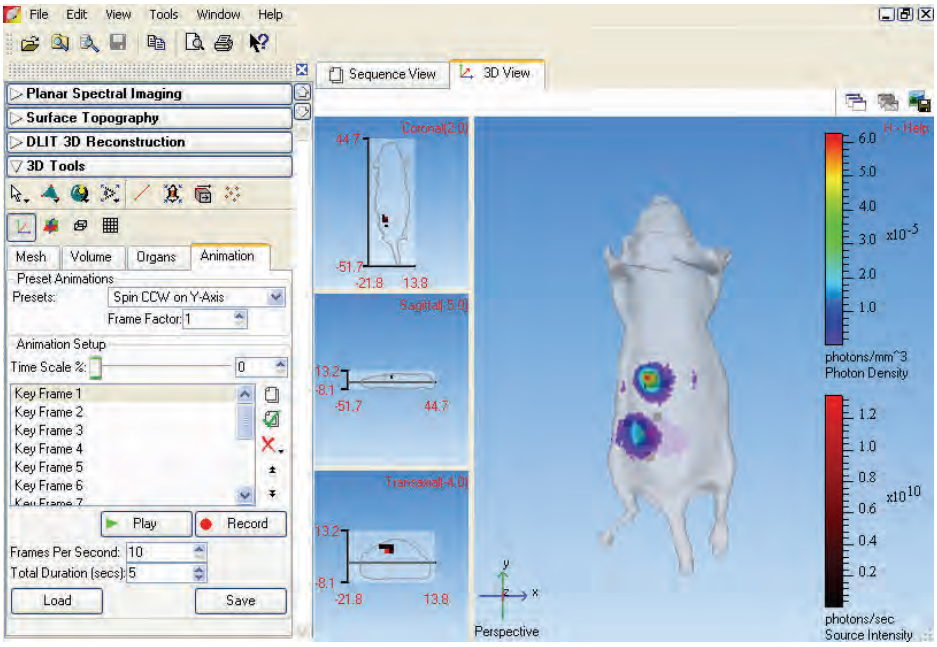
10.8 Creating an Animation

The Living Image software can produce an animation from a sequence of 3D views or *key frames*. For example, an animation can depict:

- Magnifying (zooming in on) the 3D view.
- Spinning the 3D view on an axis.
- The mesh or organs fading out (decreasing opacity) or fading in (increasing opacity).

The animation can be saved to a movie (.mov, .mp4, or .avi).

In the Animation Tab, You Can:	For More Details, See
View a preset animation (generated from a predefined animation setup)	Figure 10.29, page 144
Create a custom animation (generated from your custom animation setup)	Figure 10.30, page 145
Edit an animation setup	Figure 10.31, page 146
Save an animation to a movie	Figure 10.31, page 146



Key frame box shows the key frames in the current animation setup.

Click a key frame to display the associated 3D view and time stamp (position in the time scale (0-100) at which the frame occurs in the animation).

Figure 10.28 Animation tab

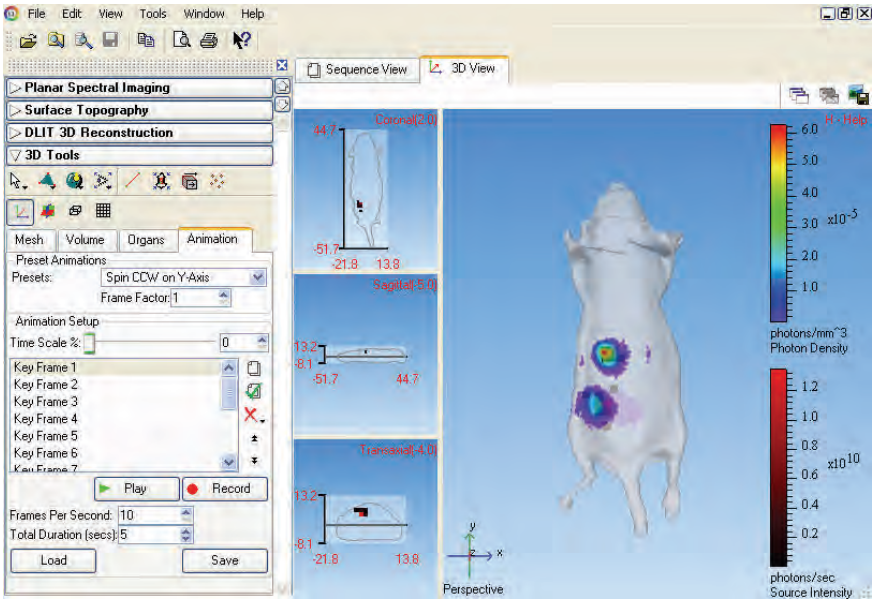
Item in the Animation Tab	Description
Time Scale%	The time stamp of a key frame in the animation on a time scale of 0-100. For example, if the animation is 10 sec long and includes five key frames: Key frame 1: Time stamp= 0; first frame of the animation. Key frame 2: Time stamp = 25%; frame occurs at 2nd sec of animation. Key frame 3: Time stamp = 50%; frame occurs at 5th sec of animation. Key frame 4: Time stamp = 75%; frame occurs at 8th sec of animation. Key frame 5: Time stamp = 100%; last frame of the animation.
Presets	A drop-down list of predefined animation setups.
Key frame	A 3D view. The software interpolates the key frames to create intermediate frames in real time, then generates an animated sequence from all of the frames. Each successive key frame in a sequence should differ slightly from the preceding one, so that motion is smoothly depicted when the frames are shown at a proper frame rate (frames/second). The Living Image software provides preset key frames or you can specify the 3D views for the key frames.
Preset Key Frame Factor	Determines how many key frames are used to generate one revolution in a spinning animation (No. of frames = (4 x Key Frame Factor) + 1). Increasing the key frame factor reduces the time period between key frames and creates the appearance of finer movement. Decreasing the key frame factor increases the time period between key frames and creates the appearance of coarser movement.
FPS	Frames displayed per second in the animation sequence.
	Click to create a new key frame from the current 3D view.
	Click to update the selected key frame to the current 3D view.
	Click to delete a selected or all key frames from the key frame box.
	Click to move a selected key frame up in the key frame box.
	Click to move the selected key frame down in the key frame box.
Total Duration	The total time of the animation sequence.
Play	Click to view the animation sequence defined by the current key frames and animation parameters.
Record	Displays a dialog box that enables you to save the current animation to a movie (.mov, .mp4, or .avi).
Animation Setup	
Load	Displays a dialog box that enables you to open an animation setup (.xml).
Save	Displays a dialog box that enables you to save the current key frames and animation parameters to an animation setup (.xml).

Viewing a Preset Animation

The software provides several predefined animations (Figure 10.29) or you can create your own. For more details on creating an animations, see Figure 10.30.

To view a preset animation:

1. Open the DLIT results of interest.
2. Confirm that the 3D view shows the properties of interest (for example, organs, voxels, mesh, or photon density maps).
3. In the 3D Tools, click the Animation tab.
4. If necessary, clear the key frame box (click the ✕ button and select **Delete All**).
5. To view a preset animation, make a selection from the Presets drop-down list. (See Table 10.7 for a description of the preset animations.)
   - A list of the key frames appears.
6. To view the animation, click **Play**.



Note: You can view multiple animations sequentially. For example, if you select Spin CW on X-Axis and Spin CW on Y-axis from the Presets drop-down list, the animation shows the 3D reconstruction spinning clockwise on the x-axis, then spinning clockwise on the y-axis.

To save the animation to a movie:

1. Click **Record**.
2. In the dialog box that appears, choose a directory and enter a file name (.mov, mp4, .avi), and click **Save**.

Figure 10.29 Viewing a preset animation

Table 10.7 Preset animations

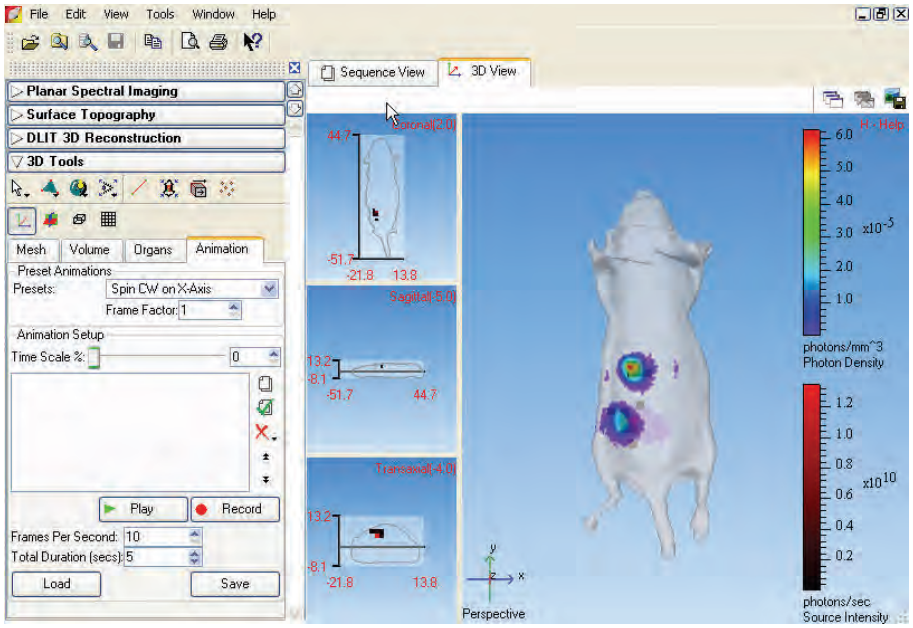
Name	Choose This Animation Setup to...
Spin CW	Rotate the 3D reconstruction clockwise.
Spin CCW	Rotate the 3D reconstruction counterclockwise.
Zoom In	Magnify the 3D reconstruction.
Fade In	Increase opacity from 0-100%.
Fade Out	Decrease opacity from 100-0%.

Creating a Custom Animation

To create an animation, you must specify a custom animation setup or edit an existing setup. For more details on editing a setup, see Figure 10.31.

To create a custom animation:

1. Open the 3D results of interest.
2. Confirm that the 3D view shows the properties of interest (for example, position or scale of the 3D reconstruction, organs, voxels, mesh, or photon density maps).
3. In the 3D tools, click the Animation tab.
4. If necessary, clear the key frame box (click the ✕ button and select **Delete All**.)
5. To capture the first key frame, click the button.
   - The first key frame is added to the key frame box.



6. To capture the next key frame, adjust the 3D view to show the properties of interest and click the button.
   - The second key frame is added to the key frame box.
7. Repeat Step 6 until all key frames of interest are captured. For details on how to edit the key frame sequence, see Figure 10.31.
   Note: Click a key frame to display the associated 3D view and the time stamp (position in the time scale (0-100) at which the frame occurs in the animated sequence).
8. Confirm the defaults for FPS (frames per second) and Total Duration (length of animation) or enter new values.
   Note: FPS x Total Duration = No. of frames generated to create the animation. The number of generated frames should be ≥ to the number of key frames. Otherwise, the frames may not be properly animated.
9. To view the animation, click **Play**. To stop the animation, click **Stop**.

To save the animation to a movie:

1. Click **Record**.
2. In the dialog box that appears, choose a directory and enter a file name (.mov, mp4, .avi), and click **Save**.

To save the animation setup:

1. Click **Save**.
2. In the dialog box that appears, select a directory and enter a file name (.xml).

Figure 10.30 Creating an animation

Editing & Saving an Animation Setup

To open an animation setup:

1. To select a predefined setup, make a selection from the Preset drop-down list.
   To select a saved user-defined setup:
   a. Click **Load**.
   b. In the dialog box that appears, select the directory and animation setup (.xml) of interest.

To edit the key frame sequence:

1. To add a key frame:
   a. Adjust the 3D view to show the properties of interest.
   b. Click the button.
2. To reorder a key frame in the sequence, select the key frame and click the or arrow.
3. To update a key frame:
   a. Select the key frame of interest.
   b. Adjust the 3D view.
   c. Click the button.
4. To delete a key frame:
   a. Select the key frame that you want to remove.
   b. Click the button and select **Delete Current**.

To save the animation setup:

1. Click **Save**.
2. In the dialog box that appears, select a directory and enter a file name (.xml).

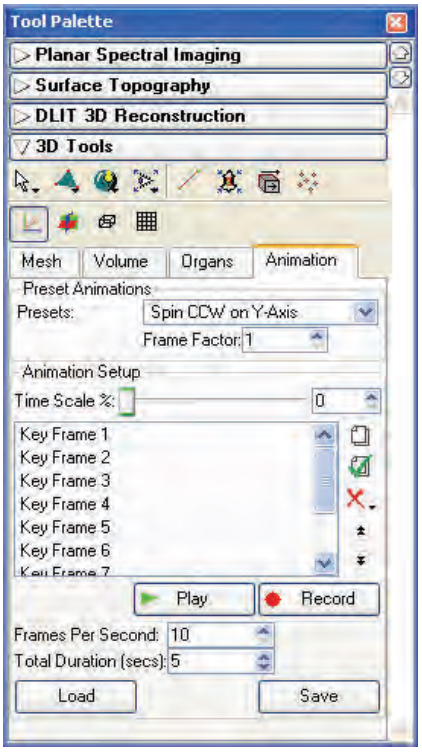


Figure 10.31 Editing and saving an animation setup

11 Biotrue Collaborative Data Management System

The Biotrue Collaborative Data Management System (CDMS) is an optional web-based application that provides secure online data storage and management capabilities.

You can upload data from the Living Image software to the Biotrue CDMS or load data from the Biotrue CDMS to the Living Image software. For more details on managing the Biotrue CDMS, see the *Biotrue CDMS User Manual*.

11.1 Uploading or Loading Image Data

To open the Biotrue browser:

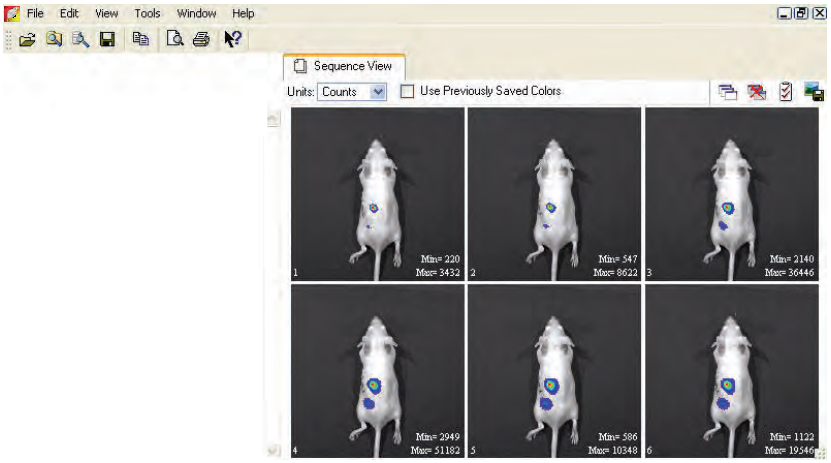
1. Click the **Browse Biotrue** button . Alternatively, select **File →Browse Biotrue** on the menu bar.

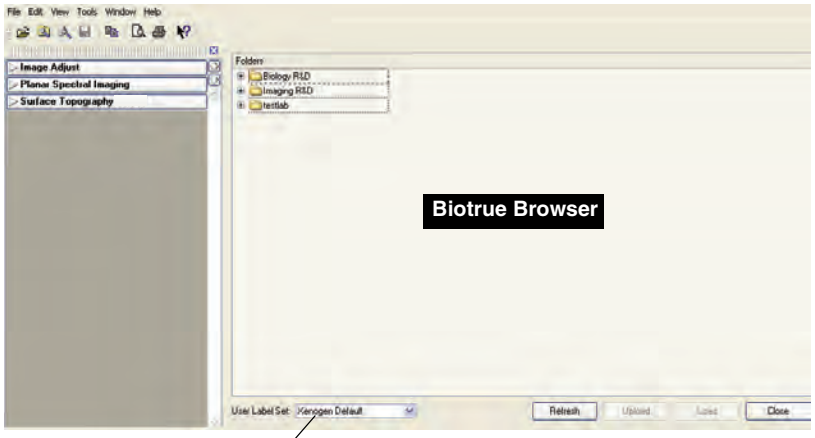
To upload data to the Biotrue CDMS:

1. Confirm that the data of interest is the active window.
2. In the Biotrue Browser, select the directory for the data and click **Upload**.
   - The image data is added to the directory.
3. Click **Refresh** to update the Biotrue browser and view new data on the Biotrue CDMS.

To load data from the Biotrue CDMS:

1. In the Biotrue Browser, select the data directory of interest and click **Load**.





Make a selection from the drop-down list to view label set information.

Figure 11.1 Uploading or downloading data from the Biotrue CDMS

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Appendix A Image Data Display & Measurement

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A.1 Image Data

Scientific Image Data

Scientific image data is a two-dimensional array of numbers. Each element of the array (*pixel*) is associated with a number that is proportional to the light intensity on the element. A charge coupled device (CCD) camera used for scientific imaging is essentially an array of photo-sensitive pixels and each pixel collects photons during an image exposure.

The subsequent electronic readout provides a photon intensity number associated with each pixel. In a bright area of the image, more photons are detected and the photon intensity number is greater than the number in a dim area of the image.

The image data can be visualized in different ways, including pseudocolor images (generated by the Living Image® software), contour plots, or isometric displays.

Graphic Image Data

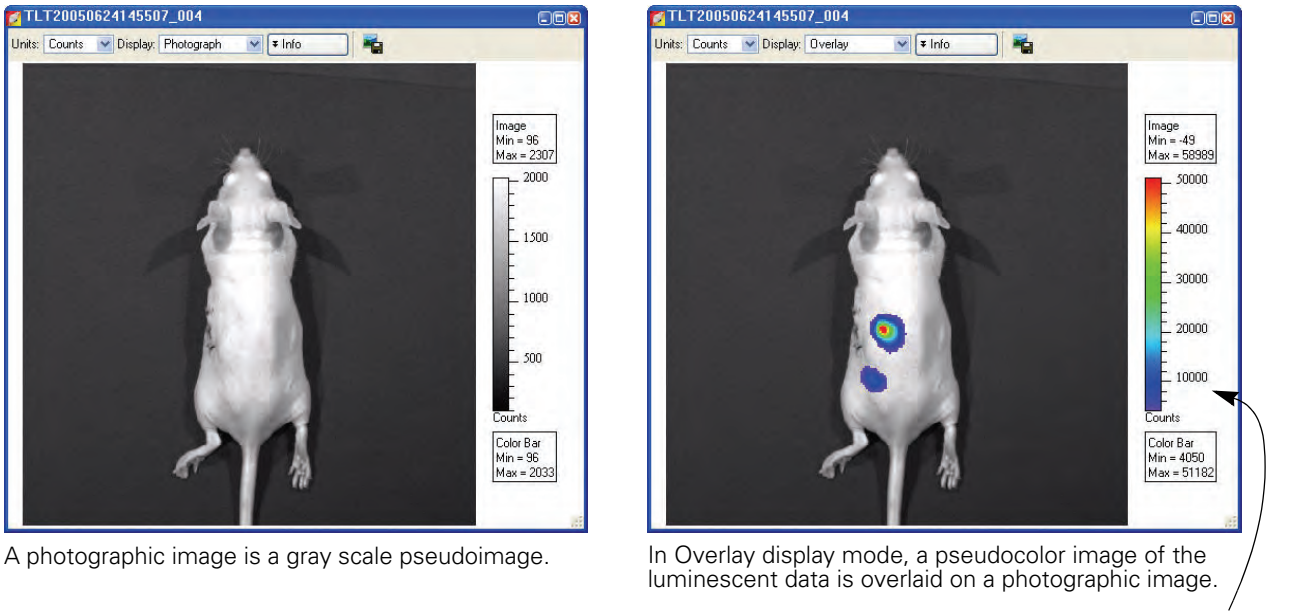
A graphic image is a two-dimensional array of pixels with a color assigned to each pixel. There are several schemes for digitally storing the images. For example, a common scheme assigns a red-green-blue (RGB) color code to each pixel. The RGB code defines how much of each color to apply in order to create the final pixel color. Color photographs or color screenshots are examples of RGB images.

An RBG image is also a two-dimensional array of numbers, but unlike a scientific image, the numbers are only color codes and are not related to light intensity. A graphic image can be exported to a graphic display application.

Pseudocolor Images

An image can be generated from scientific image data by assigning a color to each numerical value and plotting the array so that each pixel is filled with the color that corresponds to its numerical value. A *color table* defines the relationship between the numerical data and the displayed color. For example, a grayscale color table assigns black to the smallest number in the array, white to the largest number, and shades of gray to the values in between (Figure A.1). The resulting image is equivalent to a black and white photograph. An illuminated photographic image acquired on an IVIS® Imaging System is an example of a grayscale pseudoimage.

The reverse rainbow color table is also commonly used and assigns violet to the smallest number on the array, red to the largest number, and all of the spectral colors of the rainbow to the values in between (Figure A.1).



A photographic image is a gray scale pseudoimage.

In Overlay display mode, a pseudocolor image of the luminescent data is overlaid on a photographic image.

Color table associated with the data.

Figure A.1 Example pseudoimages

A pseudocolor scheme is typically used to display the numerical contents of scientific image data like the luminescent or fluorescent images acquired on an IVIS® Imaging System. The pseudocolor scheme makes it easy to see areas of bright light emission. The amount of light emission can be quantified using measurement ROIs. (For more details, *Drawing Measurement ROIs*, page 59.)

Measurement data is independent of the colors displayed in the pseudocolor image. You can change the appearance of the image data without affecting the underlying numeric pixel values. For example, you apply a different color table to the data or adjust the range of numeric values associated with the color table. Measurements that quantify pixel data produce the same results independent of the appearance of the pseudocolor display.

A pseudocolor image can be converted to an RGB color code and saved as an RGB image. The RGB image looks like a pseudocolor image, but does not include the numerical information derived from the light detected in each pixel. Therefore, the amount of light in an RGB image cannot be quantified.

Overlays

In the overlay display mode, the pseudocolor luminescent or fluorescent image is displayed on the associated grayscale photographic image (Figure A.1). Pixels in the luminescent or fluorescent image that are less than the minimum color table setting are not displayed. As a result, the lowest intensity color in the table is transparent and this enables you to view the underlying photographic image in regions where the luminescent light emission is low.

A.2 Quantifying Image Data

The Living Image software can quantify and display scientific image data using three types of measurements (Figure A.1):

- Counts
- Photons
- Efficiency (for fluorescent images only)

Data Display	Choose This to Display:	Recommended For:
Counts	An uncalibrated measurement of the photons incident on the CCD camera.	Image acquisition to ensure that the camera settings are property adjusted.
Photons	A calibrated measurement of the photon emission from the subject.	ROI measurements on bioluminescent images.
Efficiency	A fluorescence emission image normalized to the incident excitation intensity (radiance of the subject/ illumination intensity).	ROI measurements on fluorescent images.

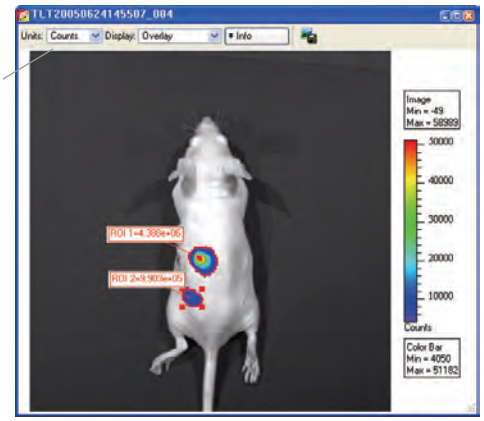
Counts

When image data is displayed in counts, the image pixel contents are displayed as the numerical output of the charge digitizer on the charge coupled device (CCD) (Figure A.2). The counts measurement (also known as *analog digitizer units* (ADU) or *relative luminescence units* (RLU)) is proportional to the number of photons detected in a pixel.

Counts are uncalibrated units that represent the raw amplitude of the signal detected by the CCD camera. A signal measured in counts is related to the photons incident on the CCD camera. The signal varies, depending on the camera settings (for example, integration time, binning, f/stop, or field of view setting).

All IVIS® Imaging Systems include a CCD digitizer that is a 16-bit device, which means that the signal count range is from zero to 65,535. Sometimes the displayed signal count may appear outside of this range due to corrections applied to the image data (for example, background corrections).

Select Counts, Photons, or Efficiency for the image data



In counts mode, the ROI measurements include:
Total Counts = Sum of all counts for all pixels inside the ROI

Average Counts = Total Counts/Number of pixels or superpixels

Quantity ROI Pixels = Number of binned pixels inside the ROI

Area (CCD pixels) = Number of unbinned CCD pixels inside the ROI

Figure A.2 Image window and ROI Measurements table (counts mode)

Photons

When image data is displayed in photons, the *photon emission from the subject* or *radiance* is displayed in photons/sec/cm²/sr. Counts are a relative measure of the photons incident on the CCD camera and photons are absolute physical units that measure the photon emission from the subject.

The radiance unit of photons/sec/cm²/sr is the number of photons per second that leave a square centimeter of tissue and radiate into a solid angle of one steradian (sr) (Figure A.3).

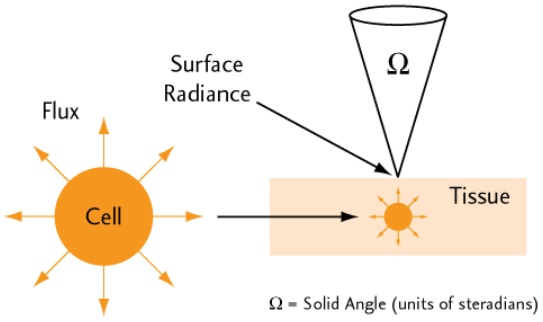


Figure A.3 Isotropic radiation
Isotropic radiation from a cell is called photon flux (photons/sec). When cells occur in tissue, photon emission from the tissue surface is called surface radiance (photons/sec/cm²/sr).

A steradian can be thought of as a three-dimensional cone of light emitted from the surface that has a unit solid angle. Much like a radian is a unit of arc length for a circle, a steradian is a unit of solid angle for a sphere. An entire sphere has 4π steradians. Lens systems typically collect light from only a small fraction of the total 4π steradians.

When image data is displayed in photons mode, the units change to photons/sec/cm²/sr. These are units of photon radiance on the surface of the animal. A very important distinction between these absolute physical units and the relative units of counts is that the radiance units refer to photon emission *from the subject animal itself*, as opposed to counts that refers to photons incident on the detector.

Measurements in units of radiance automatically take into account camera settings (for example, integration time, binning, f/stop, and field of view). As a result, images of the same subject acquired during the same session have the same signal amplitude regardless of the camera settings because the radiance on the animal surface does not change. The advantage of working with image data in photons mode is that camera settings can be changed during an experiment without having to adjust the images or the measured ROI data. Images or ROI data can be quantitatively compared across different IVIS® Imaging Systems.

Xenogen Corporation calibrates the camera settings of each IVIS Imaging System at 600 nm. The response of the CCD is relatively flat (~10%) over the range from 500-700 nm which includes the spectral variation found in bacterial or firefly luciferase. Therefore, calibration is accurate over this range.

Efficiency

The fluorescent signal detected from a sample depends on the amount of fluorophore present in the sample and the intensity of the incident excitation light. The excitation light incident on the sample stage is not uniform over the field of view (FOV). At FOV=10, there is a slightly dished illumination profile due to the close proximity of the stage to the illumination reflectors, while the

profiles for the other stage locations are peaked near their center. The illumination intensity profile varies by up to ±30% across the entire FOV (Figure A.4).

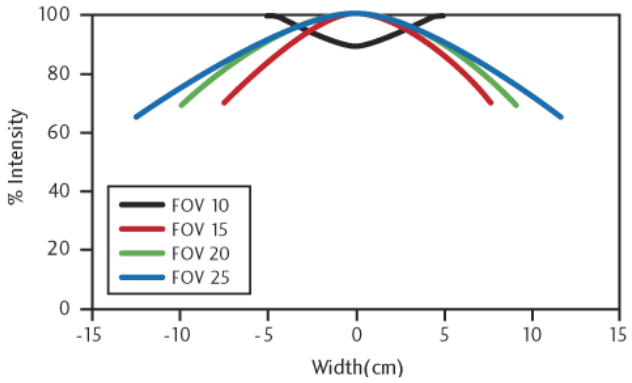


Figure A.4 Illumination profiles at different FOVs
Measurements were taken at the center of the FOV on the IVIS Imaging System 100 Series.

Displaying fluorescent image data in terms of efficiency eliminates the variable excitation light from the measurement and enables a more quantitative comparison of fluorescent signals. When you select efficiency for the image data (Figure A.2), the software normalizes the fluorescent emission image to a reference image and computes:

Efficiency = Radiance of the subject/Illumination intensity

Prior to instrument delivery, Xenogen Corporation generates a reference image of the excitation light intensity (no emission filter) incident on a highly reflective white plate for each excitation filter at every FOV and lamp power. The data are stored in the Living Image folder.

Image efficiency data does not have units. The efficiency number for each pixel represents the fraction of fluorescent photons relative to each incident excitation photon and is typically in the range of 10^{-2} to 10^{-9}. When ROI measurements are made, the total efficiency within the ROI is the efficiency per pixel integrated over the ROI area, so the resulting units of total efficiency is area or cm^2.

A.3 Flat Fielding

Flat fielding refers to the uniformity of light collected across the field of view (FOV). A lens usually collects more light from the center of the FOV than at the edges. The Living Image® 3.0 software provides a correction algorithm to compensate for the variation in the collection efficiency of the lens. This enables uniform quantitation of ROI measurements across the entire FOV.

To apply the correction algorithm, choose the Flat Field Correction option in the Corrections/Filtering tools. The algorithm multiplies each pixel by a predetermined scale factor. The scale factor for each pixel depends on its distance from the center of the image. The scale factor near the center of the field of view is one, but can be up to two or three near the corners on the IVIS® Imaging System 100 Series. (The IVIS Imaging System 200 Series has a larger lens with a smaller flat field correction.)

You may notice an increase in noise near the edges and corners of the FOV when flat field correction is applied– this is normal.

A.4 Cosmic Ray Corrections

Cosmic rays are extraterrestrial high-energy particles that register a false signal on a CCD detector. Cosmic rays as well as other sources of ionizing radiation cause infrequent interactions (a few per minute) on the CCD. These interactions result in large signals that are usually isolated to a single pixel, making them easy to correct.

The Living Image® 3.0 software searches for isolated, high amplitude *hot pixels* and replaces them with a collective average of surrounding pixels. The Cosmic Correction option should always be selected for *in vivo* image data because hot pixels can significantly affect an ROI measurement.

Cosmic ray correction is not recommended when imaging very small objects such as individual cells. An individual cell may only light up one or two pixels and can sometimes be misinterpreted as a cosmic ray. In this case, clear the Cosmic Correction option in the Corrections/Filtering tools to avoid filtering out single-cell images.

Appendix B Detection Sensitivity

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The parameters that control the number of photons collected (signal) and the image background (noise) determine the sensitivity of low light imaging. To maximize sensitivity, the goal is to increase signal and decrease background

Several factors affect the number of photons collected, including the lens f/stop, image magnification, size and detection efficiency (quantum efficiency) of the CCD, transport efficiency of the imaging optics, and the image exposure time.

B.1 CCD Detection Efficiency

IVIS® Imaging Systems use a back-thinned, back-illuminated CCD cooled to -90° to -105° C (depending on the system). This type of CCD provides high quantum efficiency of over 80% across the visible and near infrared part of the spectrum.

Figure B.1 shows detection efficiencies for several commonly used photon detectors. The back-illuminated CCD has the highest efficiency, particularly in the 600-800 nm region of the spectrum, the area of greatest interest for *in vivo* imaging.

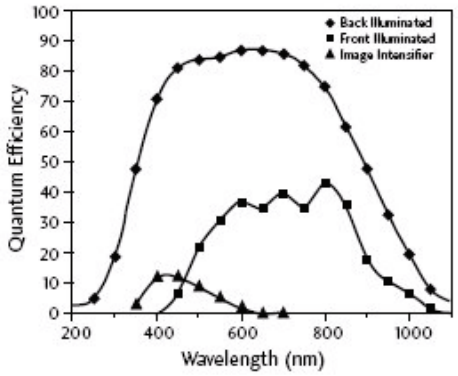

Figure B.1 Quantum efficiencies
Bialkali photocathode (▲), back-illuminated CCD (◆), and front-illuminated CCD (■). IVIS systems use back-illuminated CCDs.

Lens Aperture

IVIS® Imaging Systems are equipped with a high-light-collection f/1 lens. The sensitivity of the IVIS Imaging System can be adjusted by changing the f/stop setting that controls the lens aperture. The detected signal scales approximately as $1/(f/stop)^2$. For maximum sensitivity, select f/1, the largest aperture setting on the IVIS Imaging System (Figure B.2). This provides the greatest light collection efficiency, but results in the minimum depth of field for the image. The depth of field refers to the depth over which the image appears to be in focus and is determined by the f/stop and the field of view (FOV).

At f/1, the depth of field ranges from ~0.2 cm at FOV= 3.9 cm (IVIS® Imaging System 200 Series only) to ~2 cm at FOV= 25 cm. You can use the manual

focus option on the Control panel to easily assess the depth of field at any f/stop and FOV setting. For more details on manual focusing, see page 28. Generally f/1 is recommended for low light luminescent images and f/2 or f/4 is recommended for brighter luminescent or fluorescent images.

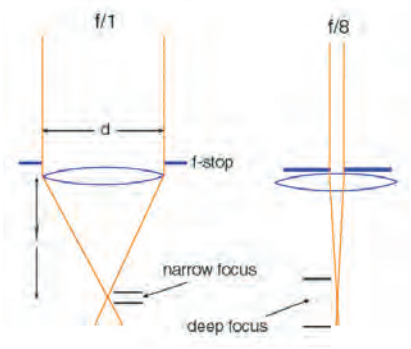

Figure B.2 Lens f/stop positions.
Left: lens wide open at f/1; right: lens closed down at f/8.

Image Exposure Time

The image exposure time also affects sensitivity. The number of photons collected is directly proportional to the image exposure time. For example, an image acquired over a two minute exposure contains twice as many detected photons as an image acquired over a one minute exposure. Longer exposure times are usually beneficial when imaging very dim samples. However, this may not always be true because some types of background, dark charge in particular, increase with exposure time. (For more details on backgrounds, see Appendix C, page 159.) An IVIS® Imaging System has extremely low background that enables exposures of up to 30 minutes. However, animal anesthesia issues and luciferin kinetics limit practical exposure times for *in vivo* imaging to 5-10 minutes.

Field of View (FOV)

The FOV indirectly affects sensitivity. Changing the FOV without changing the binning or the f/stop does not significantly affect sensitivity. However, CCD pixels are effectively smaller at a smaller FOV (higher magnification) so that higher levels of binning can be applied without loss of spatial resolution.

For example, an image acquired at binning=4 and FOV=20 cm has the same spatial resolution as an image acquired at binning=8 and FOV=10 cm. Due to the increase in binning, the latter image has a four-fold increase in sensitivity compared to the former.

B.2 Binning

A charge coupled device (CCD) is a photosensitive detector constructed in a two-dimensional array of pixels. After an image is acquired, each pixel contains an electrical charge that is proportional to the amount of light that the pixel was exposed to. The software measures the electrical charge of each CCD pixel and assigns a numerical value (*counts*). (For more details on counts and other measurement units, see Appendix A, page 149.) The resulting image data comprise a two-dimensional array of numbers; each pixel contains the counts associated with the amount of light detected.

The IVIS® Imaging Systems are equipped with a CCD that ranges from 1024× 1024 to 2048× 2048 pixels in size, and thus have a high degree of spatial resolution. At binning=1, each pixel is read and the image size (number of pixels) is equal to the physical number of CCD pixels (Figure B.3).

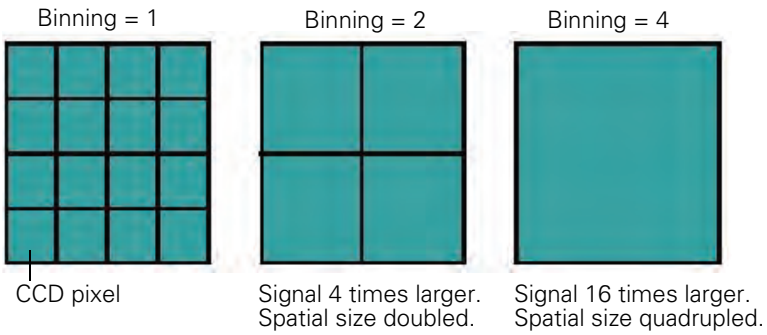

Figure B.3 A small segment of the CCD.
At binning = 2, 4 pixels are summed together; at binning = 4, 16 pixels are summed.

At binning=2, four pixels that comprise a 2× 2 group on the CCD are summed prior to read out and the total number of counts for the group is recorded (Figure B.3). This produces a smaller image that contains one fourth the pixels compared to an image at binning=1. However, due to summing, the average signal in each pixel is four times higher than at binning=1. At binning=4, 16 pixels are summed prior to read out.

Binning significantly affects the sensitivity of the IVIS Imaging System. Binning at higher levels (for example, ≥ 4) improves the signal-to-noise ratio for read noise, an electronic noise introduced into the pixel measurement at readout. If four pixels are summed before readout, the average signal in the summed pixel (*super pixel*) is four times higher than at binning=1.

The read noise for the super pixel is about the same as it was for the individual pixels. Therefore, the signal-to-noise ratio for the read noise component of the image noise is improved by a factor of four. Read noise is often the dominant source of noise in *in vivo* images, so a high binning level is a very effective way to improve the signal-to-noise ratio.

Unfortunately, binning reduces the spatial resolution in an image. For example, at binning=2, a super pixel is twice as wide as a pixel at binning=1. This results in a factor of two loss in image spatial resolution. However, for *in vivo* imaging, the added sensitivity is usually more important than the spatial resolution. Further, since *in vivo* signals are often diffuse due to scattering in tissue, little is gained by increasing spatial resolution. (For more background on the propagation of light through tissue, see *Diffusion Model of Light Propagation Through Tissue*, page 182.) In such cases, high levels of binning may be appropriate (up to 10 or 16, depending on the CCD of the IVIS® Imaging System). If signal levels are high enough that sensitivity is not an issue, then it is better to image at a lower binning level (two or four) in order to maintain a higher degree of spatial resolution.

NOTE

For application-specific questions regarding the appropriate binning level, please contact Xenogen Corporation.

The IVIS System Control panel provides several binning options. The actual binning numbers associated with these settings depends on the CCD chip and type of image (Table B.1). These choices should satisfy most user needs. However, if you want to manually control binning, you can specify **Manual Binning** in the Living Image Tools-Preference-Camera Settings box.

Table B.1 Binning settings

Binning	Camera			
	EEV	**ROPER**	**SITe**	**Andor**
Medium Lumin	Bin 8	Bin 5	Bin 4	Bin 4
Small (high-resolution) Lumin	Bin 4	Bin 2	Bin 2	Bin 2
Large (high-sensitivity) Lumin	Bin 16	Bin 10	Bin 8	Bin 8
Medium Photo	Bin 4	Bin 2	Bin 2	Bin 2
Small (high-resolution) Photo	Bin 2	Bin 1	Bin 1	Bin 1

You can also apply *soft binning* after an image is acquired. Conceptually, soft binning is the same as hardware binning—groups of pixels are summed and a smaller, lower resolution image is produced. However, in soft binning the summing is performed digitally on the stored image data, not on the electronic charge before readout as in hardware binning.

Although soft binning does not improve the signal-to-noise ratio for read noise, it may enhance the signal visibility because it reduces the statistical scatter of nearby pixel contents. Usually, hardware binning is preferred, but if it is not possible to take another image, applying soft binning to the data may provide a worthwhile solution.

B.3 Smoothing

Smoothing is a filtering method that reduces noise in the image data. To apply smoothing, the software replaces the intensity of each pixel with the average intensity of a nearby pixel neighborhood that includes the pixel. Figure B.4 shows a 3x3 pixel neighborhood.

Smoothing does not change the pixel size and helps:

- Eliminate outlier pixel values that are extremely high or low.
- Reduce noise (fluctuations) in the image to help reveal small signals.

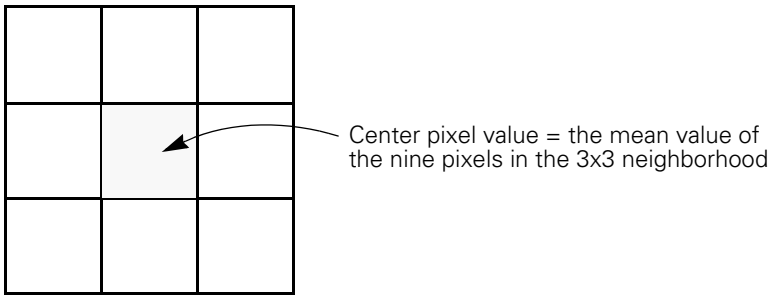

Figure B.4 3x3 pixel neighborhood

Appendix C Luminescent Background Sources & Corrections

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The background sources of light from bioluminescent images are inherently very low. This appendix discusses sources of background and how to manage them. Due to the extreme sensitivity of the IVIS® Imaging System, residual electronic background (dark current) and luminescent emission from live animals (autoluminescence) are measurable and must be taken into account.

For information on fluorescent background, see Appendix D, page 172.

C.1 Electronic Background

The cooled CCD camera used in an IVIS Imaging System has electronic background that must be accurately measured and subtracted from the image data before the light intensity is quantified. Raw data that is not corrected for electronic background results in erroneous ROI measurements. Incorrect background subtraction may also result in serious errors. However, it is not necessary to subtract the electronic background when making a simple visual inspection of an image.

The types of electronic background include:

- *Read bias* - An electronic offset that exists on every pixel. This means that the zero photon level in the readout is not actually zero, but is typically a few hundred counts per pixel. The read bias offset is reproducible within errors defined by the *read noise*, another quantity that must be determined for quantitative image analysis.
- *Dark current* - Electronic background generated by the thermal production of charge in the CCD. To minimize dark current, the CCD is cooled during use.

Read Bias & Drift

Prior to a luminescent image exposure, the Living Image® software initiates a series of zero-time exposures (*image readout*) to determine a read bias measurement.

If a dark charge background is available for the luminescent image, the average bias offset for the read bias image stored with the dark charge measurement is compared to the average bias offset determined with the read bias measurement made prior to the image. The difference, or *drift correction*, is stored with the luminescent image data, and is later used to correct minor drift (typically less than two counts/pixel) that may occur in the bias offset since measuring the dark charge background.

If a dark charge background is not available at the time of the luminescent image exposure, the software checks to see if the selected image parameters warrant a dark charge measurement (large binning and long exposure time). If a dark charge image is not required, the read bias will be used. If a dark charge is recommended, the software provides the option of using the read bias measurement instead. Since the read bias is by far the largest component of background, using a read bias measurement instead of a dark charge

measurement is often acceptable. If read bias is used instead of a dark charge background, the read bias image is stored with the image data rather than the usual background information.

If the amount of dark charge associated with an image is negligible, read bias subtraction is an adequate substitute for dark charge background subtraction. Dark charge increases with exposure time and is more significant at higher levels of binning. A good rule of thumb is that dark charge is negligible if:

$$\tau B^2 < 1000$$

where τ is the exposure time (seconds) and B is the binning factor.

Under these conditions, dark charge contributes less than 0.1 counts/pixel and may be ignored.

Dark Charge

Dark charge refers to all types of electronic background, including dark current and read bias. Dark charge is a function of the exposure time, binning level, and camera temperature. A dark charge measurement should be taken within 48 hours of image acquisition and the system should remain stable between dark charge measurement and image acquisition. If the power to the system or camera controller (a component of some IVIS Systems) has been cycled or if the camera temperature has changed, a new dark charge measurement should be taken.

The dark charge is measured with the camera shutter closed and is usually performed automatically overnight by the Living Image® software. The software acquires a series of zero-time exposures to determine the bias offset and read noise, followed by three dark exposures. The dark charge measurement usually takes more than three times as long to complete as the equivalent luminescent exposure.

C.2 Background Light On the Sample

An underlying assumption for *in vivo* imaging is that all of the light detected during a luminescent image exposure is emitted by the sample. This is not accurate if there is an external light source illuminating the sample. Any reflected light will be detected and is indistinguishable from emission from the sample.

The best way to deal with external light is to physically eliminate it. There are two potential sources of external light: a light leak through a crack or other mechanical imperfection in the imaging chamber or a source of external illumination.

IVIS® Imaging Systems are designed to be extremely light tight and are thoroughly checked for light leaks before and after installation. Light leaks are unlikely unless mechanical damage has occurred. To ensure that there are no light leaks in the imaging chamber, conduct an imaging test using the Xenogen High Reflectance Hemisphere (Figure C.1).

A more subtle source of external illumination is the possible presence of light emitting materials inside the imaging chamber. In addition to obvious sources such as the light emitting diodes (LEDs) of electronic equipment, some materials contain phosphorescent compounds.

NOTE

Do not place equipment that contains LEDs in the imaging chamber.

Phosphorescence is a physical process similar to fluorescence, but the light emission persists for a longer period. Phosphorescent materials absorb light from an external source (for example, room lights) and then re-emit it. Some phosphorescent materials may re-emit light for many hours. If this type of material is introduced into the imaging chamber, it produces background light even after the chamber door is closed. If the light emitted from the phosphorescent material illuminates the sample from outside of the field of view during imaging, it may be extremely difficult to distinguish from the light emitted by the sample.

IVIS® Imaging Systems are designed to eliminate background interference from these types of materials. Each system is put through a rigorous quality control process to ensure that background levels are acceptably low. However, if you introduce such materials inadvertently, problems may arise.

Problematic materials include plastics, paints, organic compounds, plastic tape, and plastic containers. Contaminants such as animal urine can be phosphorescent. To help maintain a clean imaging chamber, place animal subjects on black paper (for example, Artagain black paper, Strathmore cat. no. 445-109) and change the paper frequently. Cleaning the imaging chamber frequently is also helpful.

IMPORTANT

ALERT! Use only Xenogen approved cleaning agents. Many cleaning compounds phosphoresce! Contact Xenogen technical support for a list of tested and approved cleaning compounds.

If it is necessary to introduce suspect materials into the imaging chamber, screen the materials by imaging them. Acquire an image of the material alone using the same settings (for example, FOV and exposure time) that will be used to image the sample to determine if the material is visible in the luminescent image.

Microplates (white, black, or clear plastic) can be screened this way. Screen all three types with a test image. White plates appear extremely bright by IVIS® Imaging System standards and interfere with measurements. Black or clear plastic microplates do not phosphoresce, making them better choices.

The Xenogen High Reflectance Hemisphere provides a more definitive way to determine the presence of an undesirable light source (Figure C.1). It is a small white hemisphere that is coated with a non-phosphorescent material. A long exposure image of the hemisphere should produce a luminescent image in which the hemisphere is not visible.

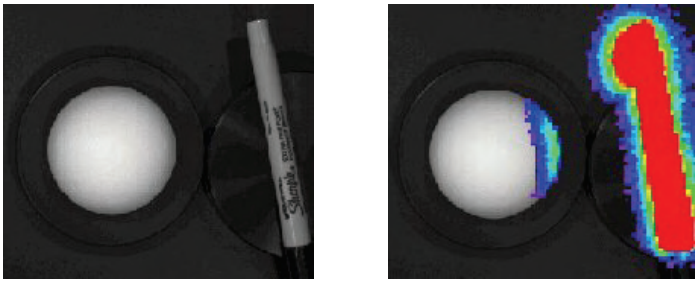

Figure C.1 Xenogen High Reflectance Hemisphere and a plastic marker pen
Left: Photographic image. Right: Photograph with luminescent overlay. The hemisphere is illuminated by phosphorescence emitted from the pen.

If any part of the hemisphere exhibits what appears to be luminescent emission, it is actually the light reflected from a source illuminating the hemisphere. Observe the side of the hemisphere that is illuminated to help determine the source location.

In Figure C.1 the pen appears very bright due to phosphorescent emission that is also illuminating the portion of the hemisphere next to the pen. If the pen had been outside the field of view, it would not have been imaged, and the source of the phosphorescence would be less obvious. However, the illumination of the hemisphere would still be very apparent and indicative of a light pollution problem.

IMPORTANT

ALERT! Handle the Xenogen High Reflectance Hemisphere by its black base plate while wearing cotton gloves provided by Xenogen. Skin oils can phosphoresce and will contaminate the hemisphere. Latex gloves and the powder on them may also phosphoresce. If the hemisphere becomes contaminated, contact Xenogen technical support for a replacement. There are no known agents that can clean the hemisphere. To check the hemisphere for contamination, take several images of the hemisphere, rotating it slightly between images. A glowing fingerprint, for example, will rotate with the hemisphere, while a glowing spot due to external illumination most likely will not.

C.3 Background Light From the Sample

Another source of background is the natural light emitted from a sample that is not due to emission from the source of interest in the sample. This type of background may be due to a material associated with the experimental setup. For example, the cell culture medium may phosphoresce. Materials should be screened so you can identify and eliminate problematic materials. If a background source is phosphorescent and the phosphorescent lifetime is relatively short, you can try keeping the sample in the dark for a long period before imaging to reduce background light emission.

Occasionally there is no way to eliminate the natural light emission of the sample. The natural light emission associated with living animals (*autoluminescence*) is a major area of interest in *in vivo* bioluminescent imaging. Most animals exhibit a low level of autoluminescence. Usually this is only a problem when looking for very low signals at the highest levels of sensitivity.

Xenogen Corporation has conducted tests to try to minimize the source of the background light emission in mice.

Test Description	Observation
Test 1: Subject animals were housed in the dark 12 hours prior to imaging.	Background emission levels were not reduced. A phosphorescent component in mouse fur or skin is not the source of light emission.
Test 2: White-furred animals were shaved prior to imaging	No increase or decrease in background emission levels.
Test 3: Alfalfa (known to be phosphorescent) was eliminated from the animal diet.	An alfalfa-free diet reduced background emission slightly, but not significantly.

The sources of autoluminescence are not yet fully understood. No external sources have been proven to cause natural light emissions, so it is possible that a chemiluminescent process associated with metabolic activity in living animals is the source of animal background. This is supported by the observation that the level of background light drops significantly in euthanized animals.

In Figure C.2 the background light emission is clearly visible in the images of a control white-furred mouse and a nude mouse. The images are five minute, high-sensitivity (high binning) exposures. The average emission from a white-furred mouse and a nude mouse is approximately 1600 photons/s/cm²/sr and 1000 photons/s/cm²/sr, respectively. Since these values are well above the lower limit of detection of the IVIS® Imaging System (~100 photons/s/cm²/sr), the background light emission from the mouse determines the limit of detection.

An approximation of this background (determined by making similar measurements on either control animals or regions of the subject animal that do not contain the primary signal) can be subtracted from ROI measurements. (For more information on ROI measurements, see Chapter 5, page 57.)

Note that the background light emission is not uniform over the entire animal. In Figure C.2 images of control animals (mice) show a somewhat higher background component originating from the abdominal and thoracic regions. Therefore, care must be taken when selecting a representative background area.

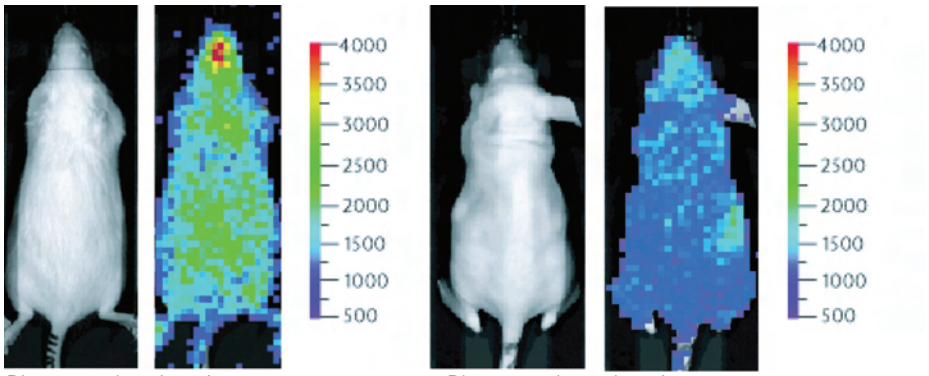

Figure C.2 Background light emission
Background light emission from a female white furred (Swiss Webster) (left) and a female nude (Nu/nu) mouse (right).

Usually only very low signals at the highest level of sensitivity require this type of background subtraction. For more information on how best to handle these types of measurements, please contact Xenogen technical support.

Appendix D Fluorescent Imaging

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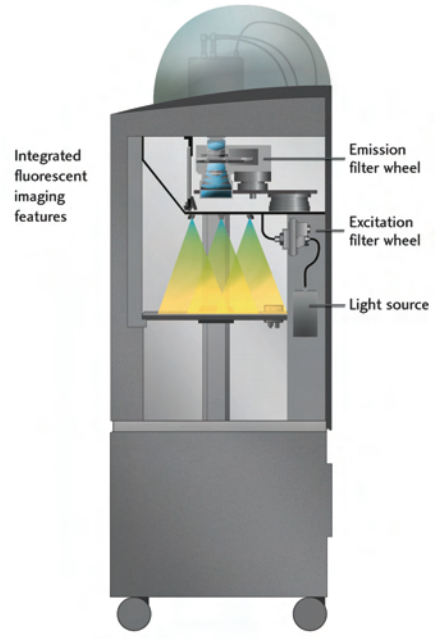
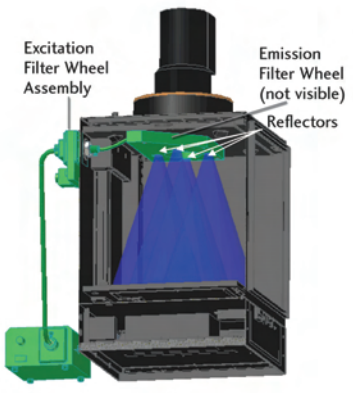
D.1 Description and Theory of Operation

System Components

The IVIS® Imaging System 200 Series and IVIS Lumina offer built-in fluorescence imaging capability as standard equipment. The IVIS Imaging System 3D, 100, or 50 Series use the XFO-6 or XFO-12 Fluorescence Option to perform fluorescence imaging. The fluorescence equipment enables you to conveniently change between bioluminescent and fluorescent imaging applications (Figure D.1). For more details, see the *IVIS Imaging System 200 Series System Manual,* the *IVIS Lumina System Manual,* or the *XFO-6* or *XFO-12 Fluorescence Option Manual.*



IVIS Imaging System 200 Series



IVIS Lumina,
IVIS Imaging System 50 or 100 Series

Figure D.1 Fluorescent imaging hardware

A 150-watt quartz tungsten halogen (QTH) lamp with a dichroic reflector provides light for fluorescence excitation. The relative spectral radiance output of the lamp/reflector combination provides high emission throughout the 400-

950 nm wavelength range (Figure D.2). The dichroic reflector reduces infrared coupling (>700 nm) to prevent overheating of the fiber-optic bundles, but allows sufficient infrared light throughput to enable imaging at these wavelengths. The Living Image software controls the illumination intensity level (off, low, or high). The illumination intensity at the low setting is approximately 18% that of the high setting.

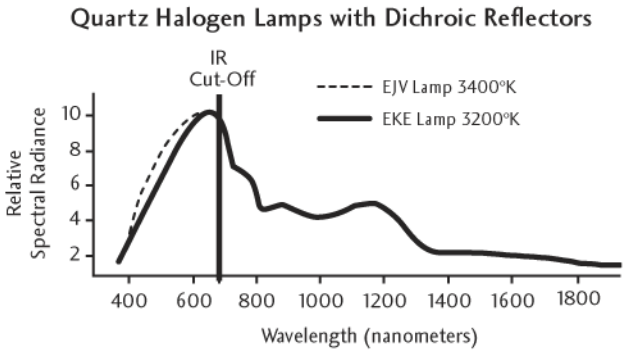


Figure D.2 Relative spectral radiance output for the quartz halogen lamp with dichroic reflector.

The lamp output is delivered to the excitation filter wheel assembly located at the back of the IVIS® Imaging System (Figure D.3). Light from the input fiber-optic bundle passes through a collimating lens followed by a 25 mm diameter excitation filter. The IVIS Imaging System provides a 12-position excitation filter wheel, allowing you to select from up to 11 fluorescent filters (five filters on older systems). A light block is provided in one filter slot for use during bioluminescent imaging to prevent external light from entering the imaging chamber. The Living Image software manages the motor control of the excitation filter wheel.

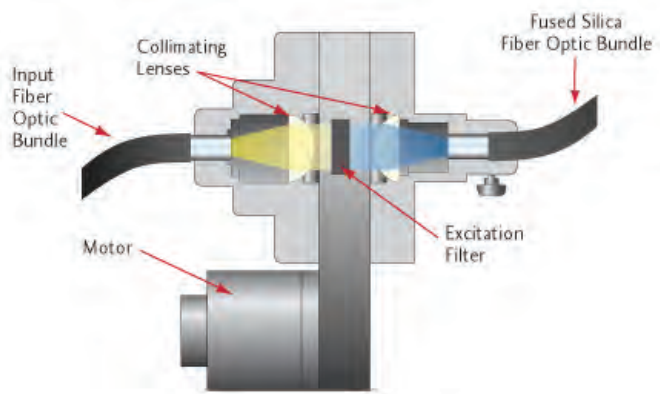


Figure D.3 Excitation filter wheel cross section.

Following the excitation filter, a second lens focuses light into a 0.25 inch fused silica fiber-optic bundle inside the imaging chamber. Fused silica fibers (core and clad), unlike ordinary glass fibers, prevent the generation of autofluorescence.

The fused silica fiber bundle splits into four separate bundles that deliver filtered light to four reflectors in the ceiling of the imaging chamber (Figure D.1). The reflectors provide a diffuse and relatively uniform illumination of the sample stage. Analyzing image data in terms of efficiency corrects for nonuniformity in the illumination profile. When the efficiency mode is selected, the measured fluorescent image is normalized to a reference illumination image. (For more details on efficiency, see page 152.)

The emission filter wheel at the top of the imaging chamber collects the fluorescent emission from the target fluorophore and focuses it into the CCD camera. All IVIS® Imaging Systems require that one filter position on each wheel always be open for bioluminescent imaging.

IVIS Imaging System	No. of Emission Filter Wheel Positions	No. of Available Fluorescence Filters
200 Series	24 (two levels, each with 12 positions)	22 (60 mm diameter)
Lumina	8	7
3D, 100 or 50	6	5 (75 mm diameter)

D.2 Filter Spectra

High quality filters are essential for obtaining good signal-to-background levels (*contrast*) in fluorescence measurements, particularly in highly sensitive instruments such as the IVIS® Imaging Systems. Figure D.4 shows typical excitation and emission fluorophore spectra, along with idealized excitation and emission filter transmission curves. The excitation and emission filters are called *bandpass filters*. Ideally, bandpass filters transmit all of the wavelengths within the bandpass region and block (absorb or reflect) all wavelengths outside the bandpass region. This spectral band is like a window, characterized by its central wavelength and its width at 50% peak transmission, or full width half maximum. Figure D.5 shows filter transmission curves of a more realistic nature.

Because the filters are not ideal, some leakage (undesirable light not blocked by the filter but detected by the camera) may occur outside the bandpass region. The materials used in filter construction may also cause the filters to autofluoresce.

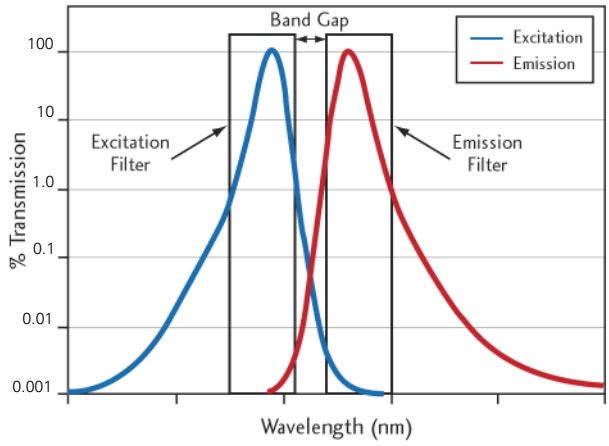


Figure D.4 Typical excitation and emission spectra for a fluorescent compound.
The graph shows two idealized bandpass filters that are appropriate for this fluorescent compound.

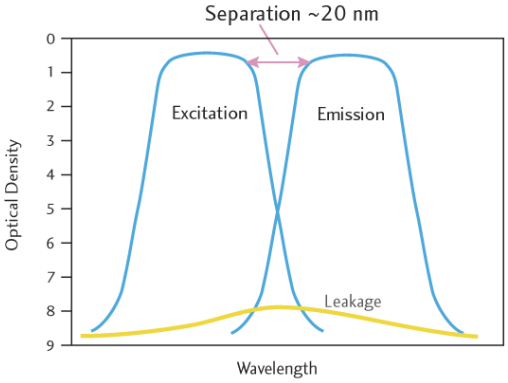


Figure D.5 Typical attenuation curves for excitation and emission filters.

In Figure D.5, the vertical axis is optical density, defined as OD = -*log*(T), where T is the transmission. An OD=0 indicates 100% transmission and OD=7 indicates a reduction of the transmission to 10^{-7}.

For the high quality interference filters in the IVIS® Imaging Systems, transmission in the bandpass region is about 0.7 (OD=0.15) and blocking outside of the bandpass region is typically in the OD=7 to OD=9 range. The band gap is defined as the gap between the 50% transmission points of the excitation and emission filters and is usually 25-50 nm.

There is a slope in the transition region from bandpass to blocking (Figure D.5). A steep slope is required to avoid overlap between the two filters. Typically, the slope is steeper at shorter wavelengths (400-500 nm), allowing the use of narrow band gaps of 25 nm. The slope is less steep at infrared wavelengths (800 nm), so a wider gap of up to 50 nm is necessary to avoid cross talk.

Fluorescent Filters and Imaging Wavelengths

Eight excitation and four emission filters come standard with a fluorescence-equipped IVIS Imaging System (Table D.1). Custom filter sets are also available. Fluorescent imaging on the IVIS Imaging System uses a wavelength range from 400-950 nm, enabling a wide range of fluorescent dyes and proteins for fluorescent applications.

For *in vivo* applications, it is important to note that wavelengths greater than 600 nm are preferred. At wavelengths less than 600 nm, animal tissue absorbs significant amounts of light. This limits the depth to which light can penetrate. For example, fluorophores located deeper than a few millimeters are not excited. The autofluorescent signal of tissue also increases at wavelengths less than 600 nm.

Table D.1 Standard filter sets and fluorescent dyes and proteins used with IVIS Imaging Systems.

Name	Excitation Passband (nm)	Emission Passband (nm)	Dyes & Passband
GFP	445-490	515-575	GFP, EGFP, FITC
DsRed	500-550	575-650	DsRed2-1, PKH26, CellTracker™ Orange
Cy5.5	615-665	695-770	Cy5.5, Alexa Fluor® 660, Alexa Fluor® 680
ICG	710-760	810-875	Indocyanine green (ICG)
GFP Background	410-440	Uses same as GFP	GFP, EGFP, FITC
DsRed Background	460-490	Uses same as DsRed	DsRed2-1, PKH26, CellTracker™ Orange
Cy5.5 Background	580-610	Uses same as Cy5.5	Cy5.5, Alexa Fluor® 660, Alexa Fluor® 680
ICG Background	665-695	Uses same as ICG	Indocyanine green (ICG)

D.3 Working with Fluorescent Samples

There are a number of issues to consider when working with fluorescent samples, including the position of the subject on the stage, leakage and autofluorescence, background signals, and appropriate signal levels and f/stop settings.

Tissue Optics Effects

In *in vivo* fluorescence imaging, the excitation light must be delivered to the fluorophore inside the animal for the fluorescent process to begin. Once the excitation light is absorbed by the fluorophore, the fluorescence is emitted. However, due to the optical characteristics of tissue, the excitation light is scattered and absorbed before it reaches the fluorophore as well as after it leaves the fluorophore and is detected at the animal surface (Figure D.6).

The excitation light also causes the tissue to autofluoresce. The amount of autofluorescence depends on the intensity and wavelength of the excitation source and the type of tissue. Autofluorescence can occur throughout the animal, but is strongest at the surface where the excitation light is strongest.

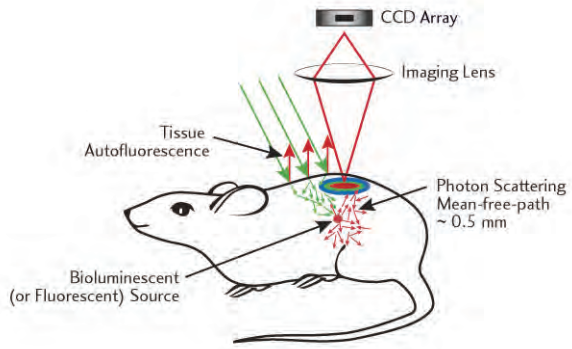


Figure D.6 Illustration of the *in vivo* fluorescence process.

At 600-900 nm, light transmission through tissue is highest and the generation of autofluorescence is lower. Therefore it is important to select fluorophores that are active in the 600-900 nm range. Fluorophores such as GFP that are active in the 450-600 nm range will still work, but the depth of detection may be limited to within several millimeters of the surface.

Specifying Signal Levels and f/stop Settings

Fluorescent signals are usually brighter than bioluminescent signals, so imaging times are shorter, typically from one to 30 seconds. The bright signal enables a lower binning level that produces better spatial resolution. Further, the f/stop can often be set to higher values; f/2 or f/4 is recommended for fluorescence imaging. A higher f/stop improves the depth of field, yielding a sharper image. For more details on the f/stop, see *Lens Aperture*, page 155.

D.4 Image Data Display

Fluorescent image data can be displayed in units of counts or photons (absolute, calibrated), or in terms of efficiency (calibrated, normalized). For more details, see *Quantifying Image Data*, page 151.

If the image is displayed in photons, you can compare images with different exposure times, f/stop setting, or binning level. When an image is displayed in terms of efficiency, the fluorescent image is normalized against a stored reference image of the excitation light intensity. Efficiency image data is without units and represents the ratio of emitted light to incident light. For more details on efficiency, see page 152.

Fluorescent Efficiency

The detected fluorescent signal depends on the amount of fluorophore present in the sample and the intensity of the incident excitation light. At the sample stage, the incident excitation light is not uniform over the FOV. It peaks at the center of the FOV and drops of slowly toward the edges (Figure D.7). To eliminate the excitation light as a variable from the measurement, the data can be displayed in terms of *efficiency* (Figure D.8).

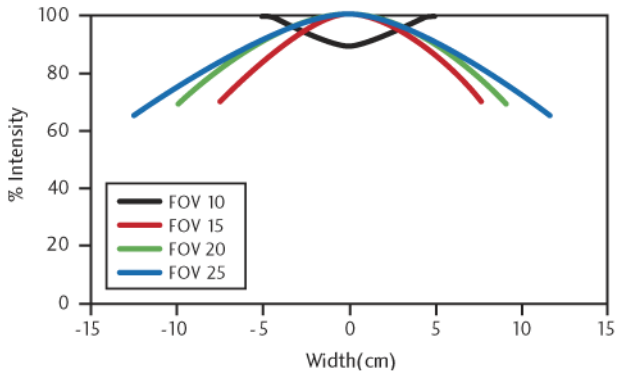


Figure D.7 Illumination profiles for different FOVs on an IVIS Imaging System 100 Series measured from the center of the FOV.

To enable a more quantitative comparison of fluorescent signals, choose Efficiency.

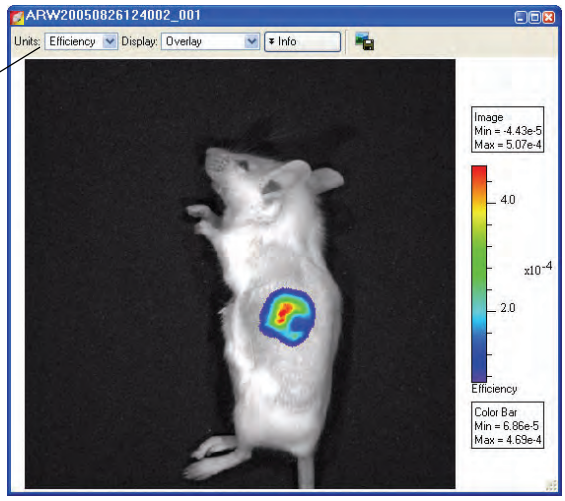


Figure D.8 Fluorescent image data displayed in terms of efficiency

When efficiency is selected, the fluorescent image data is normalized (divided) by a stored, calibrated reference image of the excitation light intensity incident on a highly reflective white plate. The resulting image data is without units, typically in the range of 10^{-2} to 10^{-9}.

NOTE

On every IVIS® Imaging System, a reference image of the excitation light intensity is measured for each excitation filter at every FOV and lamp power. The reference images are measured and stored in the Living Image folder prior to instrument delivery.

D.5 Fluorescent Background

Autofluorescence

Autofluorescence is a fluorescent signal that originates from substances other than the fluorophore of interest and is a source of background. Almost every substance emits some level of autofluorescence. Autofluorescence may be generated by the system optics, plastic materials such as microplates, and by animal tissue. Filter leakage, which may also occur, is another source of background light.

The optical components of the IVIS® Imaging Systems are carefully chosen to minimize autofluorescence. Pure fused silica is used for all transmissive optics and fiber optics to reduce autofluorescence. However, trace background emissions exist and set a lower limit for fluorescence detection.

To distinguish real signals from background emission, it is important to recognize the different types of autofluorescence. The following examples illustrate sources of autofluorescence, including microplates, other materials, and animal tissue.

Microplate Autofluorescence

When imaging cultured cells marked with a fluorophore, be aware that there is autofluorescence from the microplate as well as native autofluorescence of the cell.

Figure D.9 shows autofluorescence originating from four different plastic microplates. The images were taken using a GFP filter set (excitation 445-490nm, emission 515-575nm).

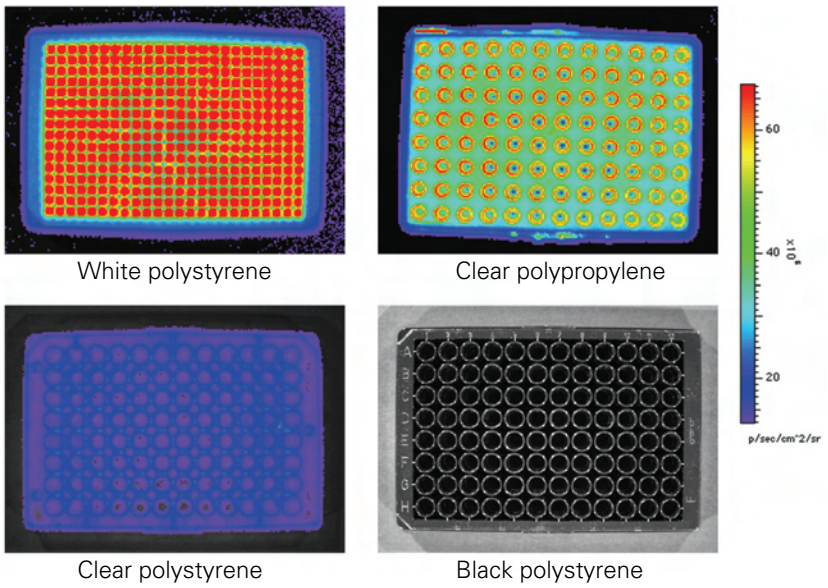


Figure D.9 Examples of microplate autofluorescence emission
The black polystyrene plate emits the smallest signal while the white polystyrene plate emits the largest signal. (Imaging parameters: GFP filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.)

Two types of autofluorescent effects may occur:

Overall glow of the material - Usually indicates the presence of autofluorescence.

Hot spots - Indicates a specular reflection of the illumination source (Figure D.10). The specular reflection is an optical illumination autofluorescence signal reflecting from the microplate surface and is not dependent on the microplate material.

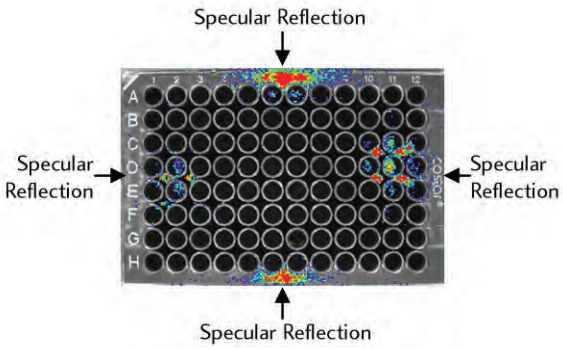


Figure D.10 Specular reflection.
The four symmetric hot spots on this black polystyrene well plate illustrate the specular reflection of the illumination source. (Imaging parameters: GFP filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.)

Black polystyrene microplates are recommended for *in vitro* fluorescent measurements. Figure D.9 and Figure D.10 show that the black polystyrene microplate emits the smallest inherent fluorescent signal, while the white polystyrene microplate emits the largest signal. The clear polystyrene microplate has an autofluorescent signal that is slightly higher than that of the black microplate, but it is still low enough that this type of microplate may be used.

Control cells are always recommended in any experiment to assess the autofluorescence of the native cell.

Miscellaneous Material Autofluorescence

It is recommended that you place a black Lexan® sheet (Xenogen part no. 60104) on the imaging stage to prevent illumination reflections and to help keep the stage clean.

NOTE

The black paper recommended for bioluminescent imaging (Swathmore, Artagain, Black, 9"x12", Xenogen part no. 445-109) has a measurable autofluorescent signal, particularly with the Cy5.5 filter set.

Figure D.11 shows a fluorescent image of a sheet of black Lexan on the sample stage, as seen through a GFP filter set. The image includes optical autofluorescence, light leakage, and low level autofluorescence from inside the IVIS® System imaging chamber. The ring-like structure is a typical background autofluorescence/leakage pattern. The image represents the minimum background level that a fluorophore signal of interest must exceed in order to be detected.

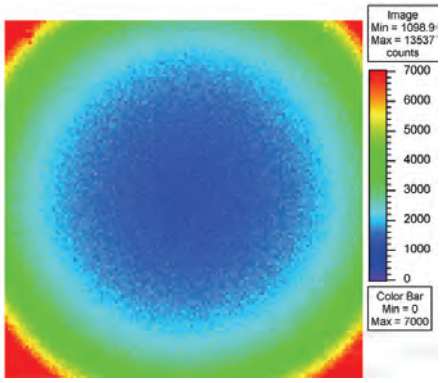


Figure D.11 Light from black Lexan

This image shows the typical ring-like structure of light from a sheet of black Lexan, a low autofluorescent material that may be placed on the imaging stage to prevent illumination reflections. (Imaging parameters: GFP filter set, Fluorescence level High, Binning=16, FOV=18.6, f/2, Exp=5sec.)

Other laboratory accessories may exhibit non-negligible autofluorescence. The chart in Figure D.12 compares the autofluorescence of miscellaneous laboratory materials to that of black Lexan. For example, the autofluorescence of the agar plate with ampicillin is more than 180 times that of black Lexan. Such a significant difference in autofluorescence levels further supports the recommended use of black polystyrene well plates.

NOTE

It is recommended that you take control measurements to characterize all materials used in the IVIS® Imaging System.

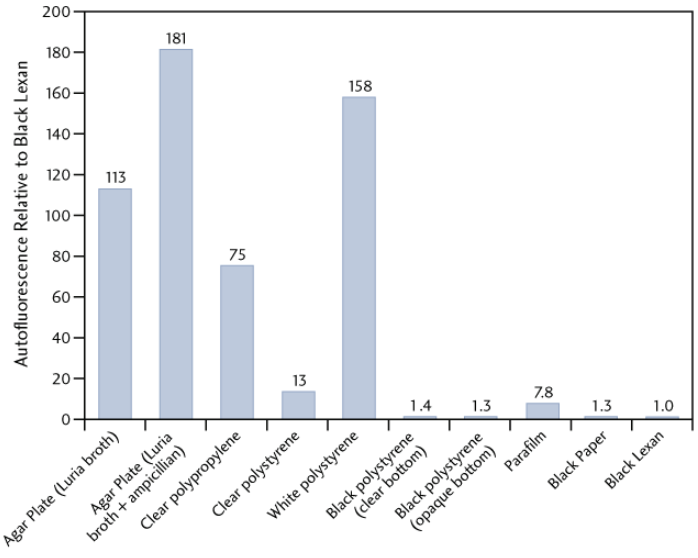


Figure D.12 Comparison of autofluorescence of various laboratory materials to that of black Lexan

Despite the presence of various background sources, the signal from most fluorophores exceeds background emissions. Figure D.13 shows the fluorescent signal from a 96-well microplate fluorescent reference standard (TR 613 Red) obtained from Precision Dynamics Co. Because the fluorescent signal is significantly bright, the background autofluorescent sources are not apparent.

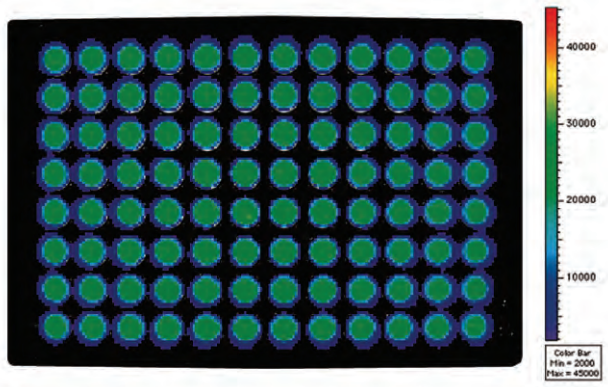


Figure D.13 96 well plate fluorescent reference standard (TR 613 Red)
The fluorescent signal is strong enough to exceed background emissions. (Imaging parameters: DsRed filter set, Fluorescence level Low, Binning=8, FOV=15, f/1, Exp=4sec.) Reference standard TR 613 Red is available through Precision Dynamics Co, http://www.pdcorp.com/healthcare/frs.html.

Animal Tissue Autofluorescence

Animal tissue autofluorescence is generally much higher than any other background source discussed so far and is likely to be the most limiting factor in *in vivo* fluorescent imaging. Figure D.14 shows ventral images of animal tissue autofluorescence for the GFP, DsRed, Cy5.5, and ICG filter set in animals fed regular rodent food and alfalfa-free rodent food (Harlan Teklad, TD97184). Animals fed the regular rodent diet and imaged using the GFP and DsRed filter sets, show uniform autofluorescence, while images taken with the Cy5.5 and ICG filter sets show the autofluorescence is concentrated in the intestinal area.

The chlorophyll in the regular rodent food causes the autofluorescence in the intestinal area. When the animal diet is changed to the alfalfa-free rodent food, the autofluorescence in the intestinal area is reduced to the levels comparable to the rest of the body. In this situation, the best way to minimize autofluorescence is to change the animal diet to alfalfa-free rodent food when working with the Cy5.5 and ICG filter sets. Control animals should always be used to assess background autofluorescence.

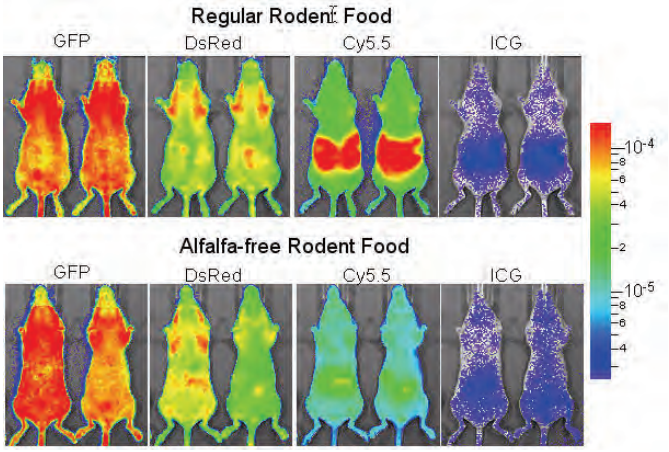


Figure D.14 Images of animal tissue autofluorescence in control mice (Nu/nu females)
Animals were fed regular rodent food (top) or alfalfa-free rodent food (bottom). Images were taken using the GFP, DsRed, Cy5.5, or ICG filter set. The data is plotted in efficiency on the same log scale.

Figure D.15 shows a comparison of fluorescence and bioluminescence emission *in vivo*. In this example, 3×10^6 PC3M-luc/DsRed prostate tumor cells were injected subcutaneously into the lower back region of the animal. The cell line is stably transfected with the firefly luciferase gene and the DsRed2-1 protein, enabling bioluminescent and fluorescent expression. The fluorescence signal level is 110 times brighter than the bioluminescence signal. However, the autofluorescent tissue emission is five orders of magnitude higher. In this example, fluorescent imaging requires at least 3.8×10^5 cells to obtain a signal above tissue autofluorescence while bioluminescent imaging requires only 400 cells.

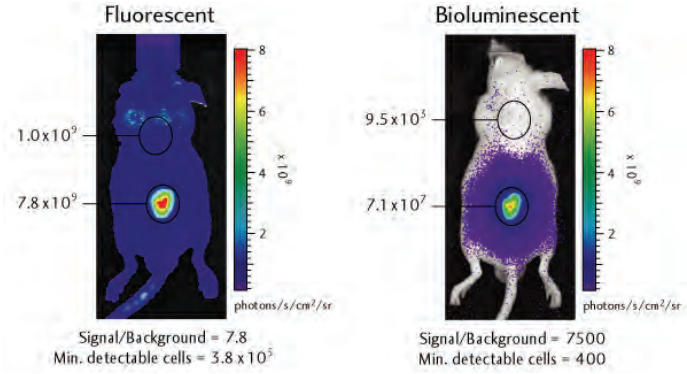


Figure D.15 Fluorescent (left) and bioluminescent (right) images of stably transfected, dual-tagged PC3M-luc DsRed cells.
The images show the signal from a subcutaneous injection of $3\text{x}10^6$ cells in an 11-week old male Nu/nu mouse.

NOTE

When you make ROI measurements on fluorescent images, it is important to subtract the autofluorescence background. For more details, see *Subtracting Tissue Autofluorescence*, page 79.

D.6 Subtracting Instrument Fluorescent Background

The fluorescence instrumentation on an IVIS® Imaging System is carefully designed to minimize autofluorescence and background caused by instrumentation. However a residual background may be detected by the highly sensitive CCD camera. Autofluorescence of the system optics or the experimental setup, or residual light leakage through the filters can contribute to autofluorescence background. The Living Image software can measure and subtract the background from a fluorescence image.

Fluorescent background subtraction is similar to the dark charge bias subtraction that is implemented in luminescent mode. However, fluorescent background changes day-to-day, depending on the experimental setup. Therefore, fluorescent background is not measured during the night, like dark charge background is.

After you acquire a fluorescent image, inspect the signal to determine if a fluorescent background should be subtracted (Figure D.16). If background subtraction is needed, remove the fluorescent subject from the imaging chamber and measure the fluorescent background (select **Acquisition →Fluorescent Background →Measure Fluorescent Background** on the menu bar). In the Living Image® software, the **Sub Fluor Bkg** check box appears on the Control panel after a background has been acquired. You can toggle the background subtraction on and off using this check box.

NOTE

The fluorescence background also contains the read bias and dark charge. Dark charge subtraction is disabled if the Sub Fluor Bkg check box is checked.

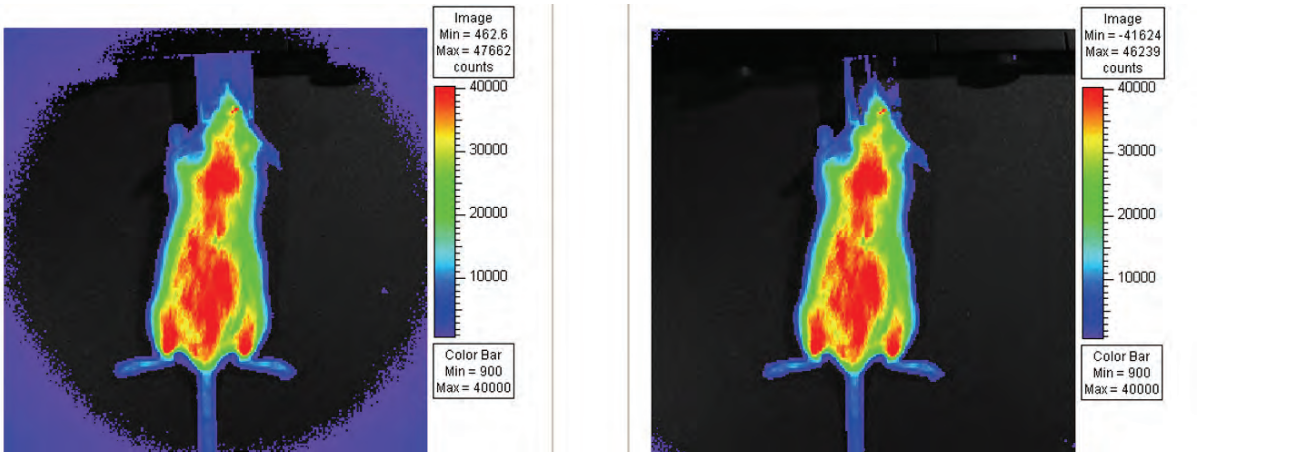


Figure D.16 Comparison of dark charge bias subtraction (left) and fluorescent background subtraction (right).
The autofluorescence from the nose cone and filter leakage have been minimized in the image on the right by using Sub Fluor Bkg option.

D.7 Subtracting Tissue Autofluorescence Using Background Filters

High levels of tissue autofluorescence can limit the sensitivity of detection of exogenous fluorophores, particularly in the visible wavelength range from 400 to 700 nm. Even in the near infrared range, there is still a low level of autofluorescence. Therefore, it is desirable to be able to subtract the tissue autofluorescence from a fluorescent measurement.

The IVIS® Imaging Systems implement a subtraction method based on the use of blue-shifted background filters that emit light at a shorter wavelength (see Table 6.2, page 79). The objective of the background filters is to excite the tissue autofluorescence without exciting the fluorophore. The background filter image is subtracted from the primary excitation filter image using the Image Math tool and the appropriate scale factor, thus reducing the autofluorescence signal in the primary image data. (For more details, see Chapter 6, page 79.) The assumption here is that the tissue excitation spectrum is much broader than the excitation spectrum of the fluorophore of interest and that the spatial distribution of autofluorescence does not vary much with small shifts in the excitation wavelength.

Figure D.17 shows an example of this technique using a fluorescent marker. In this example, 1×10^6 HeLa-luc/PKH26 cells were subcutaneously implanted into the left flank of a 6-8 week old female Nu/nu mouse. Figure D.18 shows the spectrum for HeLa-luc/PKH26 cells and the autofluorescent excitation spectrum of mouse tissue. It also shows the passbands for the background filter (DsRed Bkg), the primary excitation filter (DsRed), and the emission filter (DsRed). Figure D.17 shows the IVIS® images using the primary excitation filter, the background excitation filer, as well as the autofluorescent-corrected image.

The corrected image was obtained using a background scale factor of 1.4, determined by taking the ratio of the autofluorescent signals on the scruff of the animal. The numbers shown in the figures are the peak radiance of the animal background within the region of interest. In the corrected image, the RMS error is used to quantify the background. The signal-to-background ratio of the original fluorescent image (DsRed filter) is 6.5. The ratio increases to 150 in the corrected image, an improvement factor of 23. This improvement reduces the minimum number of cells necessary for detection from 1.5×10^5 to 6.7×10^3.

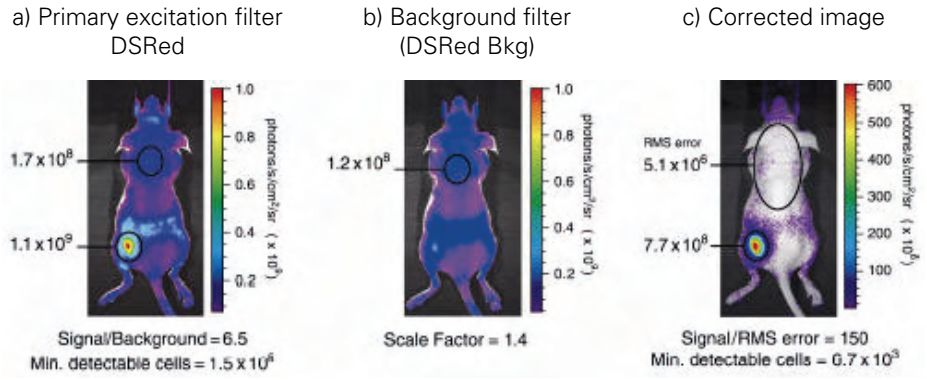


Figure D.17 Example of the autofluorescent subtraction technique using a background excitation filter.

a) primary excitation filter (DsRed), b) blue-shifted background excitation filter (DsRed Bkg), and c) corrected data. The corrected image was obtained by subtracting the scaled background filter image (multiplied by 1.4) from the primary filter image. The 6-week old female Nu/nu mouse was injected subcutaneously with 1×106 HeLa-luc/PKH2⁶ cells in the left flank.

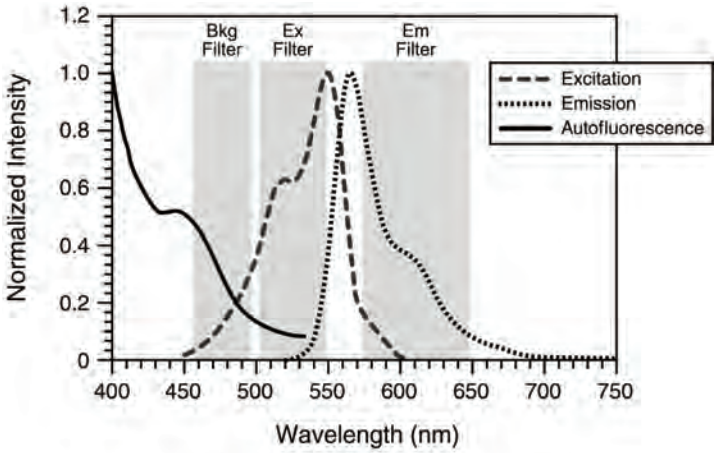


Figure D.18 Spectral data describing the autofluorescent subtraction technique using a background filter.

The graph shows the excitation and emission spectrum of PKH26 and the autofluorescent excitation spectrum of mouse tissue. Also included are the spectral passbands for the blue-shifted background filter (DsRed Bkg), the primary excitation filter (DsRed), and the emission filter used with this dye.

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Appendix E Planar Spectral Imaging

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The unique spectral signatures of the luciferase emission spectrum and the optical properties of tissue enable the Living Image software to determine the depth and intensity of light sources inside a living animal. The planar spectral imaging algorithm relies on a diffusion model of light propagation in tissue and assumes a point source of light embedded in a flat surface approximation of the mouse. The algorithm is designed to provide a fast and robust method to approximate source location and brightness. The analysis requires two or more single-view images at wavelengths between 560 and 660 nm.

The Diffuse Tomography (DLIT™) algorithm is a more complete and accurate model. It analyzes images of surface light emission to produce a three-dimensional (3D) reconstruction of the bioluminescent light sources in a subject. For more details on DLIT analysis, see Chapter 10, page 109 and Appendix F, page 189.

E.1 Planar Spectral Imaging Theory

An image acquired on an IVIS® Imaging System is a diffuse projection on the surface of the animal from the bioluminescent sources located deeper inside. Information about the depth of the bioluminescent cells can help quantify the source brightness and provide information on the location of the cells.

The Living Image software uses spectroscopic information from a single-view image to estimate the depth of the bioluminescent cells. The method takes advantage of the fact that firefly luciferase bioluminescence is emitted from 500 to 700 nm, a region of the spectrum where there are major contrasts in tissue optical properties (Figure E.1).

In this portion of the spectrum, tissue absorption drops off dramatically between 500-580 nm (green/yellow wavelengths) and 600-750 nm (red wavelengths), due mainly to the presence hemoglobin. As a result, the bioluminescent signal observed on the surface of the animal is dependent on both the wavelength and the thickness of the tissue through which it travels.

The depth and absolute photon flux of a single point source can be determined from two or more images acquired at different wavelengths using relatively simple analytical expressions derived from the diffusion model of the propagation of light through tissue.

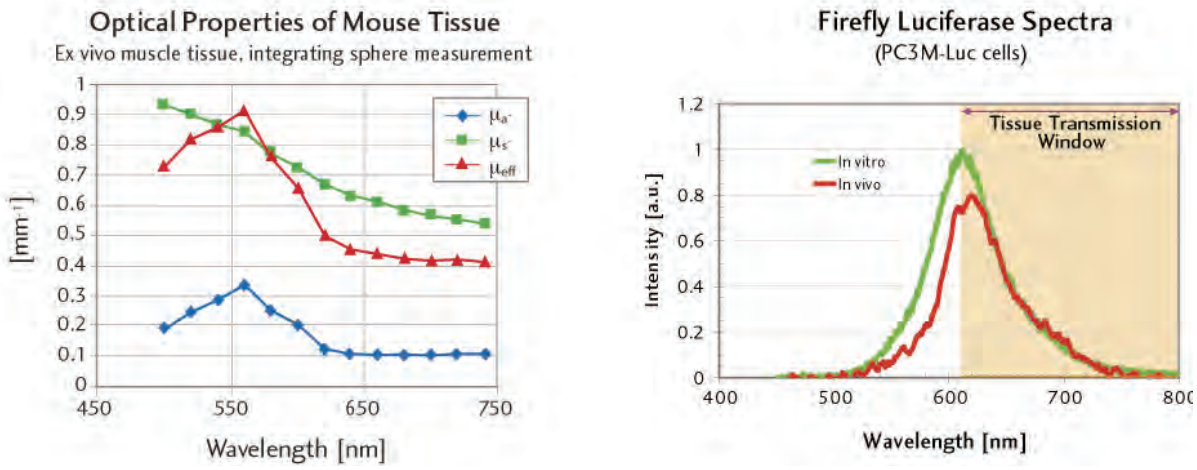

Figure E.1 Optical Properties of Mouse Tissue and Firefly Luciferase Spectra
The bioluminescent signal from firefly luciferase (right) is emitted from wavelengths of 500-700 nm, which spans a region of the spectrum where there are major contrasts in the optical properties of mouse tissue (left). The firefly spectrum was measured at 37°C using PC3M cells.

Diffusion Model of Light Propagation Through Tissue

Light propagating through tissue undergoes scattering and absorption. The diffusion model assumes that scattering is the predominant phenomenon and the reduced scattering coefficient μ'_s >> absorption coefficient μ_a. This is valid mostly for wavelengths in the red and near infrared part of the spectrum. The model also assumes that the light is produced by a single point source and that the tissues are optically homogeneous.

Under these conditions, if we model the animal surface as flat and infinite in extent and integrate the light that is collected over the animal surface, the total integrated intensity I(λ) is reduced to a relatively simple expression:

$$I(\lambda) = SK(\lambda) \exp(-\mu_{eff} d) \quad (1)$$

where S is the absolute total photon flux emitted by the bioluminescent source and d is the source depth.

The term μ_{eff} is the effective attenuation coefficient. It is determined by the tissue coefficient of absorption (μ_a) and reduced scattering (μ'_s) that quantify the two main phenomena light undergoes in tissue.

The function $K(\lambda)$ is a more complex expression that is derived from the model and includes terms that describe the effect of the tissue-air boundary on the light propagation. Both μ_{eff} and the function K are dependent on the wavelength, λ.

Equation 1 shows that if the total integrated intensity (ROI measurement) is measured at several wavelengths, it is proportional to an exponential function of the product of the depth and the optical property, μ_{eff}. Therefore, the steps to planar spectral image analysis include:

- Acquire two or more images at different wavelengths.
- Measure the total integrated intensity on each image.
- Fit the measured values to the exponential function of Equation 1.

The results of the fit are the total flux of the bioluminescence source S and the source depth d.

E.2 Optical Properties

Planar spectral image analysis requires prior knowledge of the tissue optical properties at the wavelength used at image acquisition. The two main optical parameters are the:

- *Absorption coefficient* (μ_a) that defines the inverse of the mean path before photons are absorbed by the tissue.
- *Reduced scattering coefficient* (μ'_s) that defines the inverse of the mean path before photons are scattered isotropically in the tissue.

The *effective attenuation coefficient* (μ_{eff}) is a function of the absorption and reduced scattering coefficients:

$$\mu_{eff} = (3\mu_a(\mu'_s + \mu_a))^{1/2} \quad (2)$$

Calculation of the function K in Equation 1 requires all three coefficients (μ_a, μ'_s, and μ_{eff}) as input. The function K includes a term called the *effective reflection coefficient* to account for the reflection of light at the air-tissue boundary due to a mismatch in the index of refraction. The tissue index of refraction is generally assumed to be close to 1.4.

The model assumes that the tissues are optically homogeneous and the Living Image software provides several factory set tissue optical property values to choose from.

E.3 Luciferase Spectrum

Analyzing spectrally filtered images requires knowledge of the spectral dependence of bioluminescent light emission. The luciferase bioluminescence spectrum was measured *in vitro* at 37° C and pH≈ 7 in various cell lines. This spectrum is used to normalize the photon flux values that the software measures at each wavelength.

Source spectra for several reporters are included in the database, including firefly, click beetle, renilla, and bacteria (Figure E.1).

NOTE

The firefly luciferase spectrum is temperature and pH dependent. The luciferase spectra included in the software are only valid for measurements performed at 37° C and pH 7.0-7.5. If you use other temperature or pH conditions for an experiment, the associated luciferase spectral curve is required for planar spectral image analysis. For more information on the pH and temperature dependence of the luciferase spectrum, please contact Xenogen Corporation.

E.4 An Example of Planar Spectral Imaging

Melanoma cells were injected intravenously into the tail vein of nude mice. After 13 days, metastases developed in the lungs, kidney, and hind limb bone. An image sequence was acquired on the IVIS® Imaging System 200 Series using filters at six wavelengths from 560 to 660 nm, in 20 nm intervals.

NOTE

When using the 560 nm and 580 nm band pass filters, tissue optics result in a larger attenuation of light (due mainly to hemoglobin absorption). A longer exposure time is recommended at these wavelengths.

Figure E.2 shows the metastasis sites. The signals from the lungs and right kidney are well defined in both animals. However, in the lower back area of the left mouse, the signals are in close proximity, causing an artifact in the planar spectral analysis.

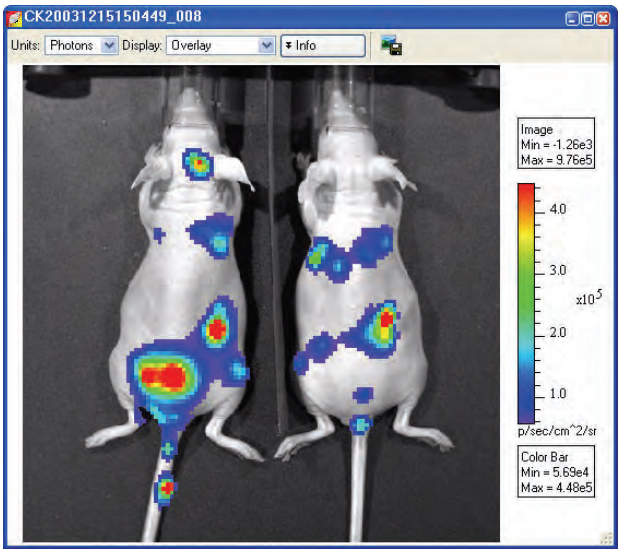

Figure E.2 Metastatic sites in nude mice.
Mice were imaged 13 days after a tail vein injection of $5x10^5$ B16F10 melanoma cells. Imaging parameters: high sensitivity binning, f/stop=1, FOV = C (13 cm), exposure time = 120 seconds at 560 and 580 nm, exposure time = 60 seconds at all other wavelengths. This resulted in signals of ~2000 counts on each image.

To perform the planar spectral analysis, draw a measurement ROI that captures the entire signal of each site of interest without including a neighboring metastasis (Figure E.3).

After the ROI is defined, start the planar spectral analysis (for more details, see *Performing Planar Spectral Image Analysis*, page 88). The software:

- Measures the total flux inside the ROI on each filtered image.
- Normalizes the data to the luciferase spectrum (Plot of Intensity vs. Lambda, Figure E.4).
- Fits the normalized data to the analytical expression in Equation 1, page 182 where S = absolute total photon flux emitted by the bioluminescence source and d = source depth (Plot of Linear Fit Results, Figure E.4)

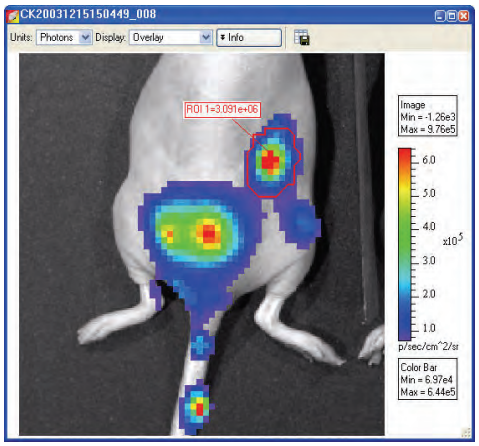

Figure E.3 Metastatic site

ROI includes the signal of the right kidney and separates it from other metastatic sites. The signal coming from the lower back area is spread out due to the presence of two bright spots. The dimmer signal in the lower bottom right of the image likely originates from the femoral bone of the animal.

After the analysis is completed, click a button to display graphical results.

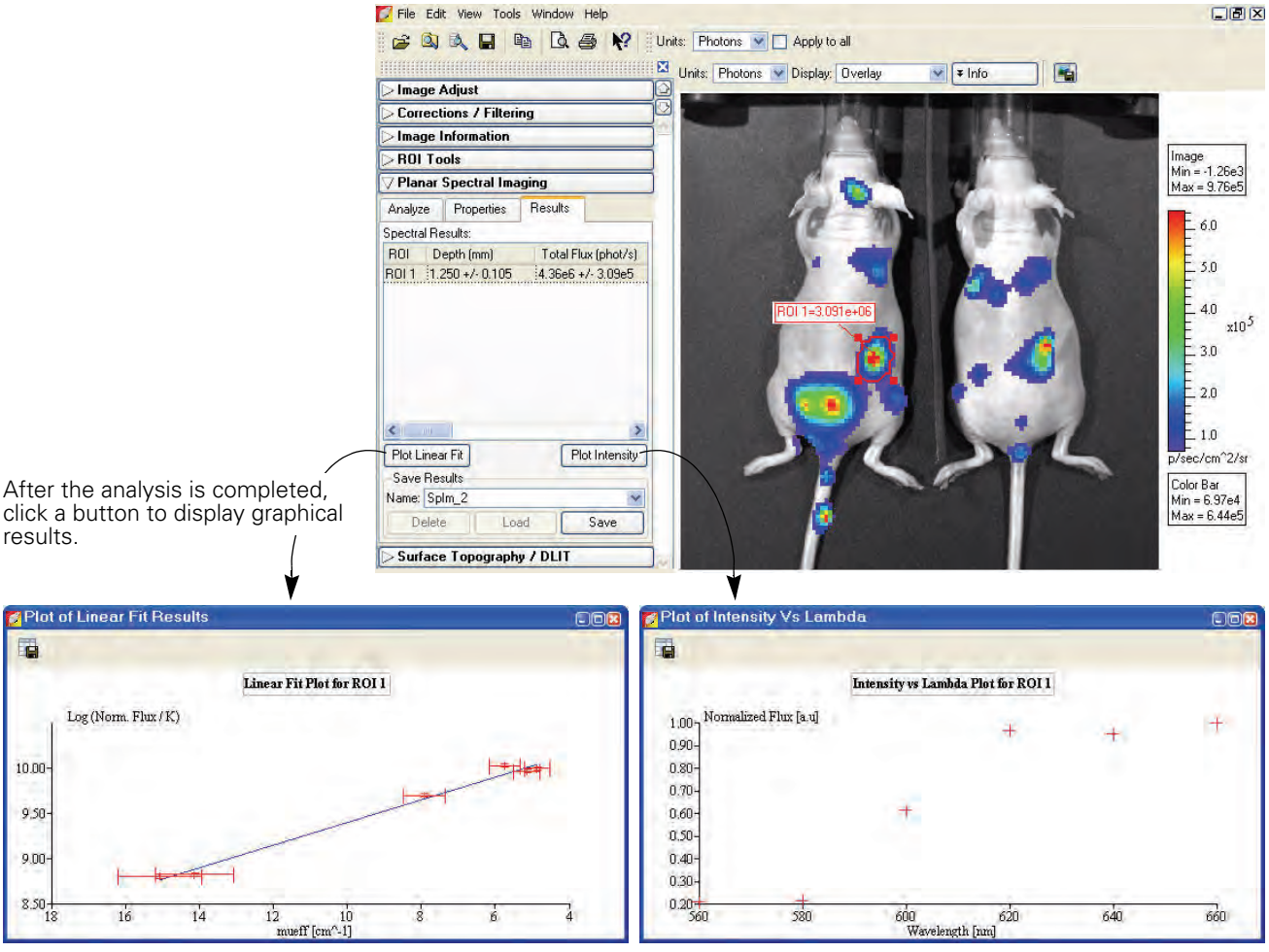

Figure E.4 Planar spectral analysis results

To estimate the cell count, divide the absolute photon flux by the flux per cell. This is best determined by making independent *in vitro* measurements of the cell line used in the experiment.

The Plot of Linear Fit Results is weighted by the uncertainty of the measured images and takes into account the uncertainty in the determination of the optical properties. The precision of the method is largely determined by the known precision of the optical properties. In most cases, the relative uncertainty in the depth determination is equal to the relative uncertainty in the optical properties.

An analysis of the dorsal and ventral views of the mouse left lung in Figure E.5 results in total flux values that are very similar. The measured depth values are also close, indicating that the cells are distributed about the same distance from the front and back of the animal.

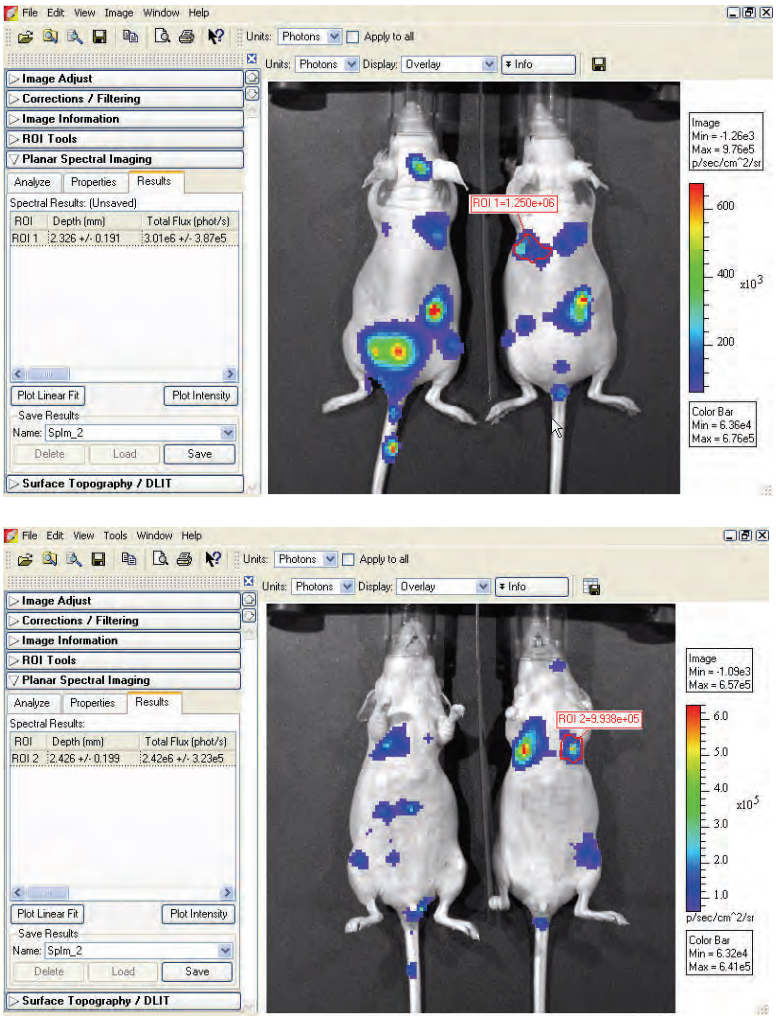

Figure E.5 Planar spectral analysis results
Top: Dorsal view of the left lung, bottom: ventral view of the left lung

E.5 Optimizing the Precision of Planar Spectral Analysis

The accuracy of the planar spectral analysis is highly dependent on the quality of the:

- Measured data for the firefly luciferase spectrum and the tissue optical properties.
- Fit of the experimentally measured total flux at each wavelength to μ_{eff} (effective attentuation coefficient).

In general, more experimental values produce a better fit of the data. It is particularly important to be able to extract signals at all wavelengths to optimize the quality of the fit. If the software detects no signal above the animal background level at 560 nm and 580 nm (the wavelengths that absorb the most light), the dynamic range of the optical properties is reduced and with it, the precision of the fit.

If a bioluminescent signal is dim or buried deep in the tissue, it may barely exceed the tissue autoluminescence at the shorter, more absorbing wavelengths (560 and 580 nm). In this case, it is recommended that you subtract the tissue autoluminescence from the image data. (For more details on subtracting tissue autoluminescence, see Appendix C, page 162). It is also recommended that you inspect all images in the sequence to confirm that the bioluminescent signal is greater than the tissue autoluminescence. If the bioluminescent signal does not exceed the tissue autoluminescence at a particular wavelength, do not include that wavelength in the analysis.

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Appendix F 3D Reconstruction of Light Sources

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Diffuse Tomography (DLIT) is a technique that analyzes images of the surface light emission from a living subject to generate a three-dimensional (3D) reconstruction of bioluminescent light source distribution inside the subject.

Fluorescent Tomography (FLIT) analyzes images of surface light emission to generate a 3D reconstruction of fluorescent light source distribution inside the subject.

NOTE

To generate a 3D reconstruction of bioluminescent sources, the Living Image software requires a photographic image, a structured light image, and bioluminescent images obtained at two or more wavelength filters from 560-660 nm. To generate a 3D reconstruction of fluorescent sources, the software requires a structured light and fluorescent images obtained using the same excitation and emission filters at different transillumination source positions on the IVIS Spectrum.

To localize and quantify the light sources in a subject, the software:

- Reconstructs the subject surface topography (*mesh*) from structured light images. The mesh is defined by a set of connected polygons or surface elements.
- Maps the surface radiance (photons/s/cm^2/steradian) to the photon density (photons/mm^3) just beneath the surface of each element of the mesh.
- Divides the interior of the subject into a solid mesh of volume elements or *voxels*. Each voxel is considered to contain a point light source at its center that contributes to the photon density at each surface element.
- Defines equations that relate the source strength of each voxel to the photon density at each surface element.
- Determines the optimum approximate solution to the system of linear equations to reconstruct the source strength in each voxel.

F.1 Determining Surface Topography

The software determines the surface topography or mesh from a structured light image. Parallel laser lines are projected onto the subject to produce a structured light image (Figure F.1).

NOTE

If the Structure option is chosen in the Control panel, a structured light image is automatically acquired.

The surface topography of the subject is determined by analyzing the displacement (Δx) or bending of the laser lines as they pass over the subject. The displacement is defined as the difference between where the line should fall on

the stage in the absence of the subject and where it appears in the image due to occlusion by the subject.

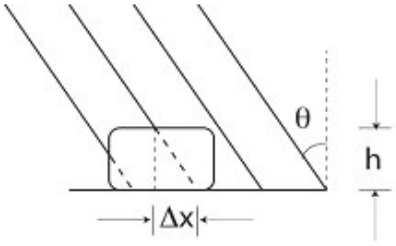

Figure F.1 Parallel laser lines projected onto a subject.
Given knowledge of the angle θ, the height of the subject (h) can be determined by analyzing the displacement, Δx, of the laser lines as they pass over the object.

The parallel lines are projected onto the surface of the subject at an angle (θ). The angle is known by instrument calibrations of the distance between the structured light projector and the optical axis (*D*) and the distance between the stage and the structured light projector (*l*) (Figure F.2).

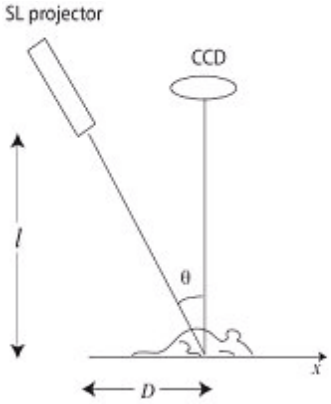

Figure F.2 Structured light projector and subject.

D and *l* form two perpendicular sides of a triangle giving:

$$\tan \theta = D/l$$

Together Δx and *h* comprise a smaller version of this triangle. The height (*h*) can be determined from:

$$h = \Delta x / \tan \theta$$

by measuring the displacement Δx.

The software utilizes fast numerical methods to rapidly evaluate Δx over the entire image to determine the surface topography. The surface topography determination is limited to the topside of the object facing the lens.

Converting Light Emission to a Photon Density Map

The input data to the FLIT algorithm for 3D reconstruction of fluorescent light sources includes:

- A surface mesh that defines the surface of the subject.
- A sequence of images acquired at different transillumination source positions using the same excitation and emission filter at each position.

The input data to the DLIT algorithm for a 3D reconstruction of bioluminescent light sources includes:

- A surface mesh that defines the surface of the subject.
- A sequence of two or more images of the light emission from the surface of the subject acquired at different filter bandwidths (Table F.1)

Table F.1 Filter center frequencies and bandwidths

IVIS® Imaging System	Filter Center Frequency (nm)	Bandwidth (nm)
3.0 Series	570	35
	600	35
	660	80
200 Series	560	20
	580	20
	600	20
	620	20
	640	20
	660	20

The IVIS® Imaging System 3D and 200 Series, and the IVIS Spectrum are absolutely calibrated so that the electron counts on each CCD pixel can be mapped back to the surface of the object to produce an absolute value of the surface radiance (photon/s/cm²/steradian) from each imaged surface element (Figure F.3).

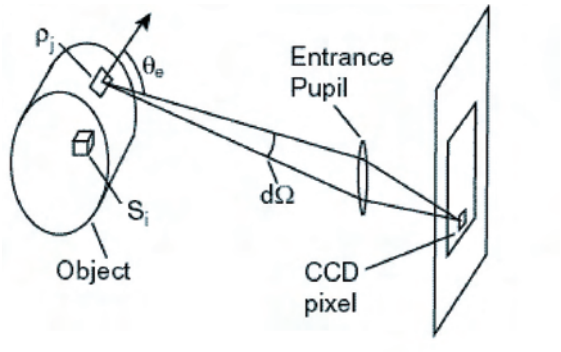

Figure F.3 Light emission from a surface element passes through the lens entrance pupil and is recorded in the image.

The imaging system collects the light emitted from the surface element at an angle (θ_e) (measured with respect to the normal to the surface element) into the solid angle $d\Omega$ subtended by the entrance pupil. The value of the surface

radiance $L(\theta_e)$ is directly related to the photon density ρ (photons/mm^3) just inside the surface of the element.

Defining the Linear Relationship Between a Source and Photon Density

The software divides the interior of the subject into a solid mesh of volume elements (*voxels)*. Each voxel is considered to contain a point light source at its center. The index i enumerates the set of voxels. S_i is the value of the strength (or flux in photons/sec) of the point source inside the i^{th} voxel. The solid mesh defines a collection of point sources that approximate the actual source distribution. The accuracy of the approximation is improved by increasing the density of the solid mesh.

The reconstruction method is based on the principle that there is an approximately linear relationship between the source strength in each voxel (S_i) and the photon density (ρ_j) at each surface element described by a Green's function G_{ij}. The photon density at the j^{th} surface element is the sum of the contributions from all the voxels:

$$\rho_j \cong \sum_i G_{ij} S_i \tag{1}$$

The Green's function contains information about the transport of photons through the tissue and the effects of the tissue-air boundary. By using a planar boundary approximation, the Green's function can be calculated analytically as a solution to the diffusion equation. Having an analytic expression for G allows Equation 1 to be computed very rapidly.

Finding the Best Approximate Solution to the Linear System

Once the Green's functions, G_{ij}, are known, the goal is to solve Equation 1 for the source strength S_i in each voxel. The DLIT algorithm attempts to minimize χ^2 (Equation 2) while requiring that the source strength in each voxel is positive (Equation 3).

$$\chi^2 = \sum_j \frac{1}{\sigma_j^2} \left[\rho_j - \sum_i G_{ij} S_i \right]^2 \tag{2}$$

$$S_i \geq 0 \tag{3}$$

A combination of methods called Simplex and Non-Negative Least Squares are used to find the approximate solution which minimizes χ^2. In order to reduce the number of variables in the problem, the code only uses surface elements with signal above a certain threshold (minimum radiance) and only keeps the voxels that contribute significantly to these surface elements.

Source & Tissue Properties

DLIT analysis of spectrally filtered images requires knowledge of the spectral dependence of bioluminescent light emission. Table F.2 shows the factory set source spectra provided by the software.

NOTE

The source spectra is not an input to the 3D reconstruction of fluorescent sources.

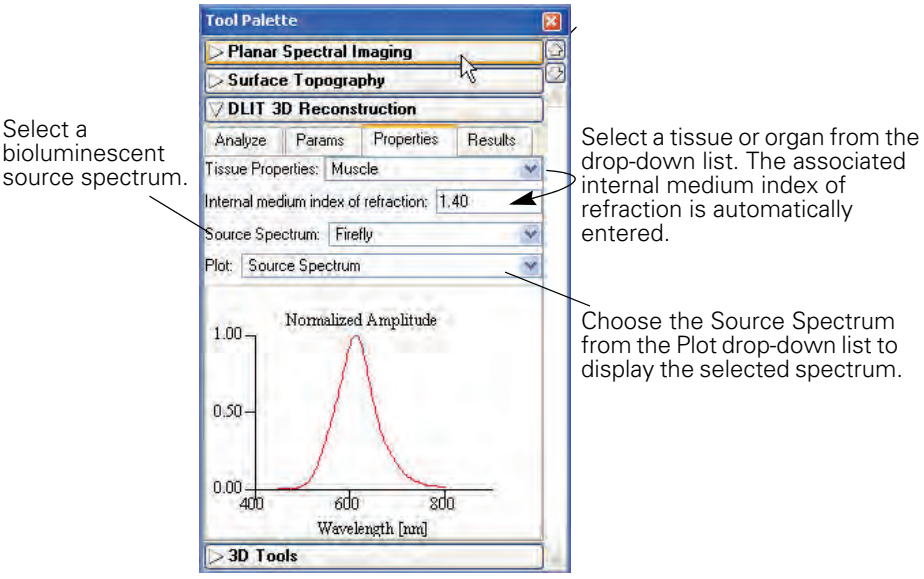

Figure F.4 DLIT 3D reconstruction tools, Properties tab

Table F.2 Source spectra

Source Spectrum	Description
Bacteria	Bacterial luciferase
CB Green	Click beetle green luciferase
CB Red	Click beetle red luciferase
Firefly	Firefly luciferase
XPM-2-LED	LED in the XPM-2 mouse phantom.
hRenilla	Sea pansy (*Renilla reniformis*) luciferase

NOTE

The firefly luciferase spectrum is dependent on temperature and pH. The data provided are valid only for measurements performed at 37° C and at pH 7.0-7.5. Selecting other temperature and pH conditions for a specific experiment requires the use of the associated spectral curve for the spectral analysis. For more information about pH and temperature dependence of the luciferase spectrum, please contact Xenogen Corporation.

You can view tissue optical property values (μ_{eff}) in the Tissue Properties drop-down list. The tissue properties are plotted as a function of wavelength. Select the tissue or organ most representative of the source location. Fat or muscle are good choices for general reconstructions.

NOTE

Default tissue optical properties and source spectrum are specified in the Preferences box. For more details, see Appendix H, page 207.

F.2 Algorithm Parameters & Options

This section explains the user-modifiable DLIT algorithm parameters and options.

Analyze Tab

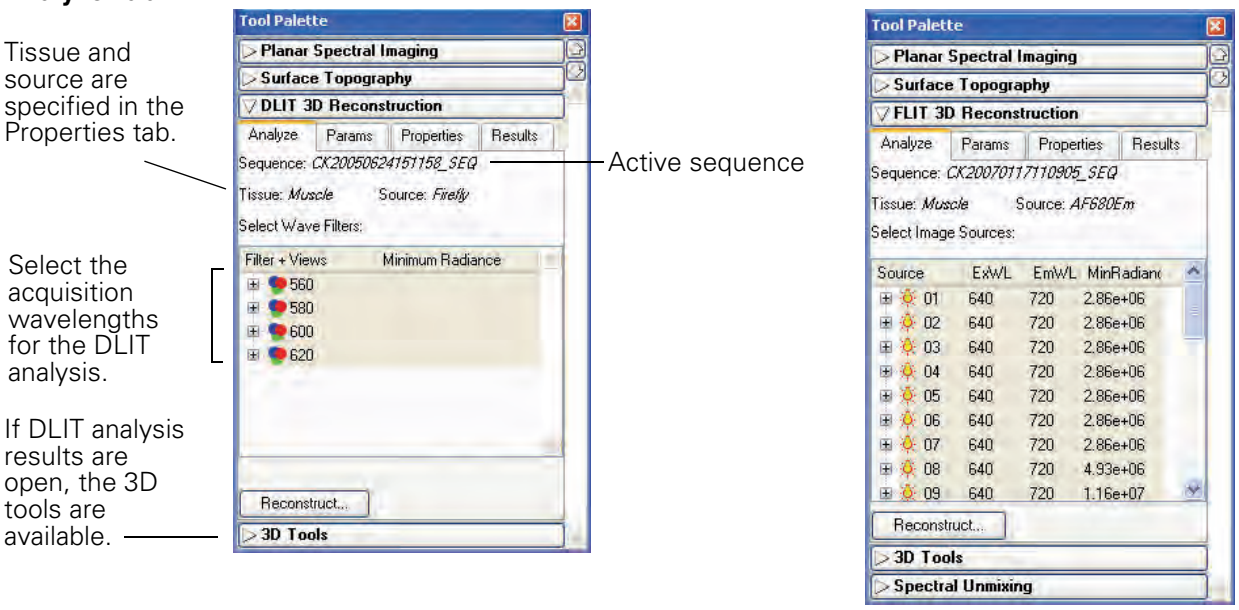

Figure F.5 3D reconstruction tools, Analyze tab, DLIT (left) and FLIT (right)

Wavelengths

For FLIT reconstruction of fluorescent sources, you must specify the transillumination source positions. It is recommended that you acquire images at a minimum of four source positions. (All images are acquired using the same excitation and emission filters.)

For DLIT reconstruction of luminescent sources, you must specify the acquisition wavelengths for the image sequence. It is generally recommended that you acquire image data using two to four wavelengths rather than a single wavelength so that more information is available for the analysis.

Ideally, chose wavelengths or source positions where the signal is well above zero (not buried in the CCD noise) and the optical property of the medium (μ_{eff}) exhibits a large change. The larger the difference in μ_{eff}, the higher the quality of information that the wavelength data adds to the analysis. The recommended

wavelengths are 580-640 nm for tissue and 560-620 nm for the Xenogen XPM-2 tissue phantom.

Minimum Radiance

The minimum radiance determines the lower radiance [photons/sec/cm²/sr] threshold of the data to be used in the DLIT analysis.

The software automatically computes a default minimum radiance value and this is the recommended starting point for an analysis. If too much noise is included in the analysis, increase the minimum radiance value.

An optimum minimum radiance value can be evaluated by viewing the image data in photon units (photons/sec/cm²/sr) and adjusting the color bar Min to be above the level of noise in the image.

Parameters Tab

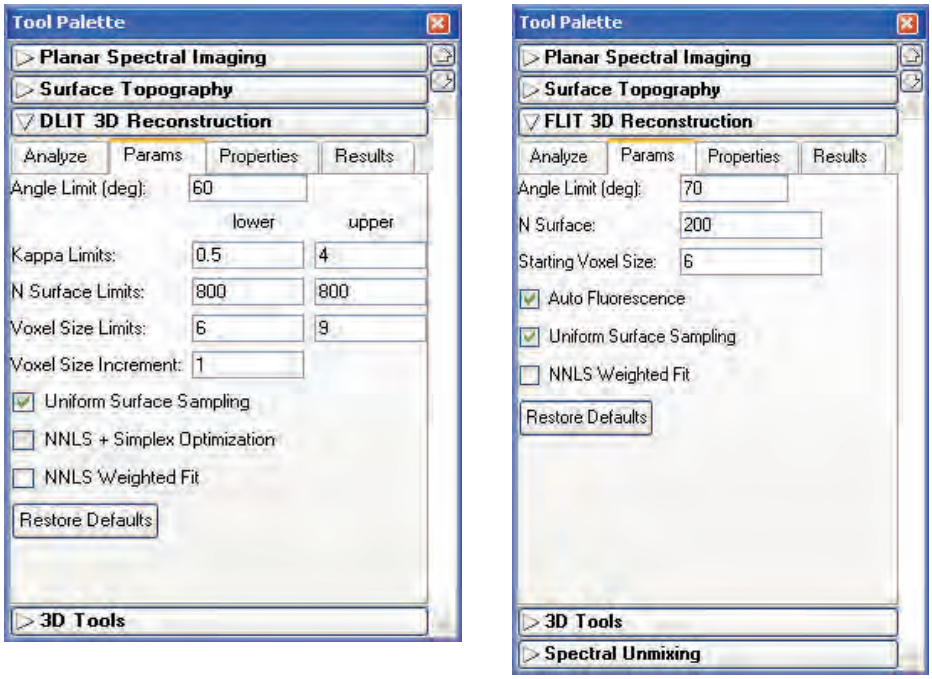

Figure F.6 3D reconstruction tools, Parameters tab, DLIT (left) and FLIT (right)

Angle Limit

The angle limit refers to the angle between the object surface normal and the optical axis. The optical axis can be considered to be a line perpendicular to the stage. The surface normal is a line perpendicular to a plane tangent to the surface point. For example, in a dorsal view of a mouse, the highest point on its back would have a normal line perpendicular to the stage. In this case the angle is zero. The side of a mouse abdomen would have a normal line parallel to the stage, so the angle here would be close to 90° .

The software uses luminescent image data for surface elements that are less than the angle limit. The default angle limit setting is 70° for the IVIS® Imaging System 200 Series or IVIS Spectrum and 60° for the IVIS Imaging System 3D Series. For IVIS Spectrum or 200 Series data, if there is significant signal on the side of the subject, a larger angle of 70-85° can be used.

Kappa Limits

Kappa (κ) is a parameter that is searched during a reconstruction to determine the best fit to the image data. Small values of kappa tend to favor deeper sources, while large values favor more shallow sources.

The limits on kappa are minimum of 0.1 and a maximum of 10. The default range for kappa is 0.5-4. Kappa is doubled at each iteration, so for a selected range of 0.5-4, the kappa values for each iteration would be 0.5, 1, 2. and 4. Choosing a large range for kappa produces the most reliable solution, but requires more analysis time.

N Surface (FLIT)

The number of surface intensity points to use in the reconstruction at a given source position.

N Surface Limits (DLIT)

This is the maximum number of surface intensity points to use in the reconstruction at a given wavelength. The range is 200 to 800 and the default is 200. The time required for reconstruction is shortest for smaller values of N (for example, 200). However, a large N value may give a more accurate result because more data are included in the fit.

Starting Voxel Size (FLIT)

Voxels are the small cubes of space inside a subject, each of which contains a quantity of fluorescent yield. The FLIT reconstruction begins with large voxels, specified by the starting voxel size (the length of a voxel cube side in mm). At each iteration, the algorithm reduces the size of the voxels by a factor of 2 until the optimum solution is determined.

Starting Voxel Size Limits (DLIT)

Voxels are the small cubes of space inside a subject, each of which contains a light source (much like a pixel in a 2D image). The DLIT reconstruction begins with large voxels, specified by the voxel size limit (the length of a side of the voxel cube in mm). At each iteration, the algorithm reduces the size of the voxel by a factor of two until the optimum solution is found.

The voxel size limits are a minimum of five and a maximum of 10. The default range is set to 6-9 mm. A larger range of voxel limits ensures a more reliable solution, but requires more computational time.

Voxel Size Increment (DLIT)

This is the step increment in voxel size, stepping from the minimum voxel size limit to the maximum voxel size limit. For example, if the voxel size limit ranges from 6-9 mm, a voxel size increment = 1 gives four starting voxel sizes (6, 7, 8, and 9 mm).

The default increment of 1 mm is usually adequate, however smaller increments can be used if you want to sample finer voxel sizes. Smaller increments will significantly increase the time required for reconstruction.

Autofluorescence (FLIT)

Choose this option to take the autofluorescence signal into account. Autofluorescence and fluorophore emission contribute to the photon density signal at the surface. The autofluorescence signal is modelled in order to isolate the signal due to the fluorophore only, where an average homogenous tissue autofluorescence yield is determined experimentally.

Autofluorescence contribution to the photon density at the surface is forward-modelled. Simulated photon density data due to autofluorescence at the surface is subtracted from the measured photon density so that the subsequent photon density used in the fit consists only of signal that is associated with the fluorophore.

Uniform Surface Sampling

If this option is chosen, the surface data for each wavelength will be sampled spatially uniformly on the signal area. If this option is not chosen, the maximum 'N surface elements' will be sampled for the data. This means that the N brightest surface elements will be used as data in the reconstruction. Typically, non-uniform sampling is recommended if there is a single bright source, while uniform sampling is preferred if there are several scattered sources.

NNLS Optimization + Simplex Optimization (DLIT)

If NNLS Optimization + Simplex option is chosen, the software uses a linear programming algorithm to seed the solution, followed by the NNLS optimization.

NNLS Weighted Fit

Choose this option to weight the data in the NNLS optimization.

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Appendix G Menu Commands & Tool Bar

Figure G.1 Living Image toolbar

Menu Bar Command	Toolbar Button	Description
File → Open		Displays the Open box so that you can select and open an image data file.
File → Browse		Displays the Browse For Folder box so that you can select and an image data folder. The selected folder is displayed in the Living Image browser.
File → Browse Biotrue		Opens the Biotrue® CDMS Browser. **Note:** The browser is only available if the system includes the Biotrue CDMS option.
File → Save		Saves (overwrites) the active image data.
File → Save As		Displays the Browse For Folder box so that you can specify a folder in which to save the image data. The original data is not overwritten.
File → Import →Organ Atlas		Opens a dialog box that enables you to import an organ atlas (.atlas).
File → Import → DICOM		Opens a dialog box that enables you to import .dcm image data that can be viewed in the Living Image software.
File → Import → 3D Mesh		Opens a dialog box that enables you to import a mesh (.xmh). **Note:** This command is only available if an appropriate sequence is active (DLIT or planar spectral imaging sequence).
File → Import → 3D Volume		Opens a dialog box that enables you to import a source volume (voxels, .xsc). **Note:** This command is only available if an appropriate sequence is active (DLIT or planar spectral imaging sequence).
File → Export DICOM		Opens the Browse for Folder dialog box that enables you to export the active image data to DICOM format (.dcm).
File → Export → 3D Mesh		Opens a dialog box that enables you to save the 3D mesh of the active data in Open Inventor format (.iv).
File → Export → 3D Volume		Opens a dialog box that enables you to save the voxels from the active data in Open Inventor format (.iv).
File → Print		Displays the Print box.
File → Print Preview		Displays the Print Preview box that shows what will be printed.
File → Recent Files		Shows recently opened data sets. Note: The number of files displayed can be set in the Preferences box (select **Edit →Preferences** and click the Customize tab).
File→ Exit		Closes the Living Image software.
Edit → Copy		Copies the active image window to the system clipboard.

Menu Bar Command	Toolbar Button	Description
Edit → Image Labels		Opens the Edit Image Labels dialog box that enables you to edit the label set information for the active data.
Edit → Preferences		Opens the Preferences box.
View → Tool Bar		Choose this option to display the toolbar.
View → Status Bar		Choose this option to display the status bar at the bottom of the main window.
View → Activity Window		Displays the Activity window at the bottom of the main application window. The Activity window shows a log of the system activity.
View → Tool Palette		Choose this option to display the tool palette.
View → Activity Window		Choose this option to display the activity bar at the bottom of the main window. The activity bar lists a history of the recent software activities.
View → Image Information		Displays the Image Information box that shows the label set and image acquisition information for the active data.
View → ROI Properties		Displays the ROI Properties dialog box.
View → ROI Measurements		Displays the ROI Measurements table.
View → Image Layout Window		Opens the Image Layout window that enables you to paste an image of the active data in the window.
View → Acquisition Control Panel		Displays the control panel.
Acquisition → Background → Replace		Opens a dialog box that enables you to select an instrument luminescent background. This background measurement is subtracted from luminescent images.
Acquisition → Background → View Available Dark Charge		Opens a dialog box that enables you to view the dark charge measurements for the system.
Acquisition → Background → Clear Available Dark Charge		Opens a dialog box that enables you to remove the dark charge measurements from the system.
Acquisition → Background → Measure		Opens a dialog box that enables you to acquire a dark charge measurement.
Acquisition → Fluorescent Background → Add or Replace Fluorescent Background		Opens a dialog box that enables you to select an instrument fluorescent background measurement for the active image data. If the Sub Fluor Bkg option is chosen in the control panel, the background measurement is subtracted from the image data.
Acquisition → Fluorescent Background → Measure Fluorescent Background		Starts a measurement of the instrument fluorescent background.
Acquisition → Fluorescent Background → Add or Replace Fluorescent Background		Opens a dialog box that enables you to select a fluorescent background measurement.
Acquisition → Fluorescent Background → Clear Available Fluorescent Background		Opens a dialog box that enables you to remove the fluorescent background measurements from the system.
Acquisition → Fluorescent Background → View Available Fluorescent Background		Opens a dialog box that displays the fluorescent background measurements for the system. If a fluorescent background is selected, the Sub Fluor Bkg option appears in the control panel. Choose the Sub Fluor Bkg option to subtract the user-specified background measurement from the image data.
Tools → Image Math for...		Opens the Image Math window for the active data.
Tools → Image Overlay for...		Opens the Image Overlay window for the active data.
Window → Close		Closes the active image window.
Window → Close All		Closes all image windows.

Menu Bar Command	Toolbar Button	Description
Window → Cascade		Organizes the open image windows in a cascade arrangement (Figure 4.13, page 42).
Window → Tile		Organizes the open image windows in a tiled arrangement (Figure 4.13, page 42).
Window → 1. xx **Window → 2. xx** **Window → etc.**		A list of the open data. Select the data of interest to it the active window and display it on top of all other open windows.
Window → Other Windows → Browser Window		If the Living Image browser is open, makes it the active window and displays it on top of all other open windows.
Help → User Guide		Displays the online help.
Help → About Living Image		Displays the online help index.
		Click this button, then click an item in the user interface to display information about the item.

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Appendix H User Preferences

Viewing User Preferences 203
Managing User IDs 210

You can manage user IDs and specify defaults for some preferences that are associated with the user ID selected at the start of a new session. User-modifiable preferences include the:

- Appearance of the application window (Figure H.1).
- Image color settings.
- Folder path setting for exporting image data.
- Number of recently viewed data sets to include in the Recent Files menu.
- Default tissue properties and DLIT algorithm parameters.
- Host and user settings for the optional Biotrue Collaborative Data Management System.

H.1 Viewing User Preferences

■ To view the user-modifiable settings, select **Edit →Preferences** on the menu bar.

If you have not yet logged on to the system, the Select User ID box appears. Select a user ID or enter a new ID, and click **OK**.

— The Preferences box appears (Figure H.1).

NOTE

Any changes made in the Preferences box are implemented at the start of the next session.

General Preferences

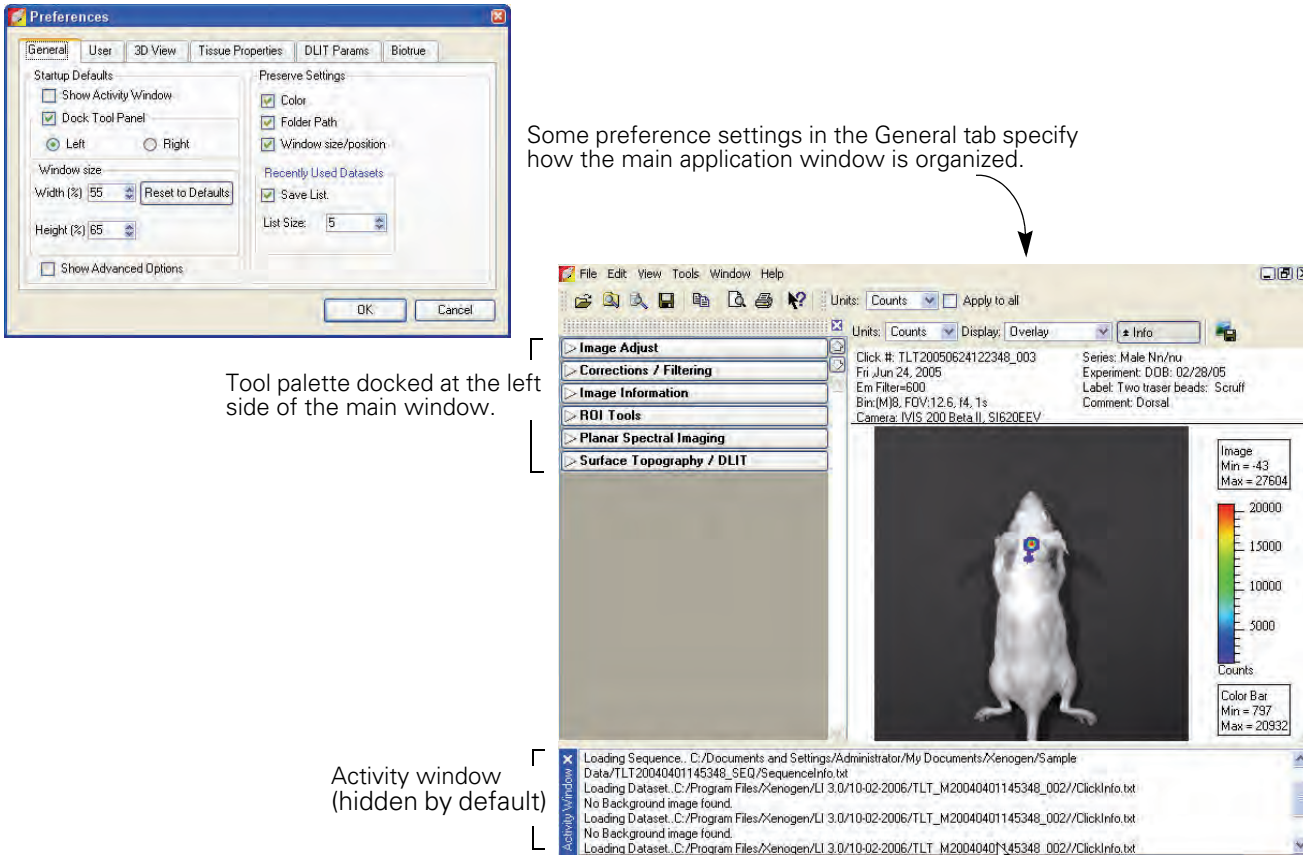


Figure H.1 Preferences, General tab (top) and main application window (bottom)
The main application window contains the image window, tool palette, and activity window.

Preferences, General Tab	Description
Startup Defaults	
Show Activity Window	Displays the activity window at the bottom of the application window.
Dock Tool Panel	Sets the position of the tool palette in the application window. Choose left or right.
Window Size	Specifies the dimensions of the main application window.
Width, Height	Sets the dimensions of the image window.
Default	Click to apply the default settings.
Preserve Settings	
Color	Applies the color settings of the active image data to subsequently opened image data.
Folder Path	Sets the default folder path to the current folder path setting. Click the **Export** button in the image window to view the current folder path setting (Figure H.1).
Window size/position	Applies the active image window size and position settings to subsequently opened image data.
Recently Used Datasets	
Save List	Displays recently opened files on the Recent Files menu.
List Size	Enter a number or click the up/down arrows to specify the number of files to display.

User Preferences

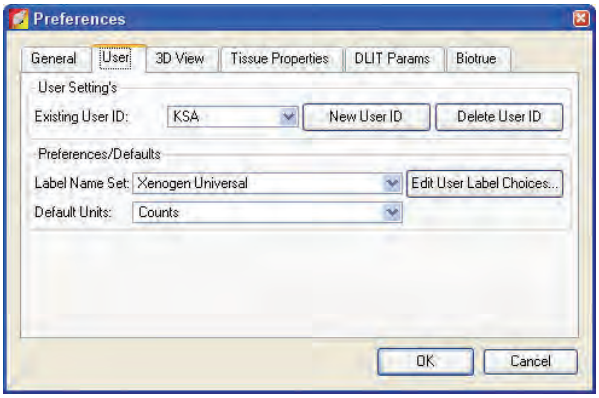


Figure H.2 Preferences, User tab

Preferences, User Tab	Description
User settings	
Existing User ID	Drop-down list of users.
New User ID	Opens the Add New User box. A new user is added to the Existing User ID drop-down list.
Delete User ID	Deletes the user selected from the Existing User ID drop-down list.
Preferences/Defaults	
Label Name Set	Drop-down list of factory installed label name sets.
Edit User label Choices	Opens a dialog box that enables you to edit a label set.
Default Units	Specifies the units (photons or counts) for image display.

3D View

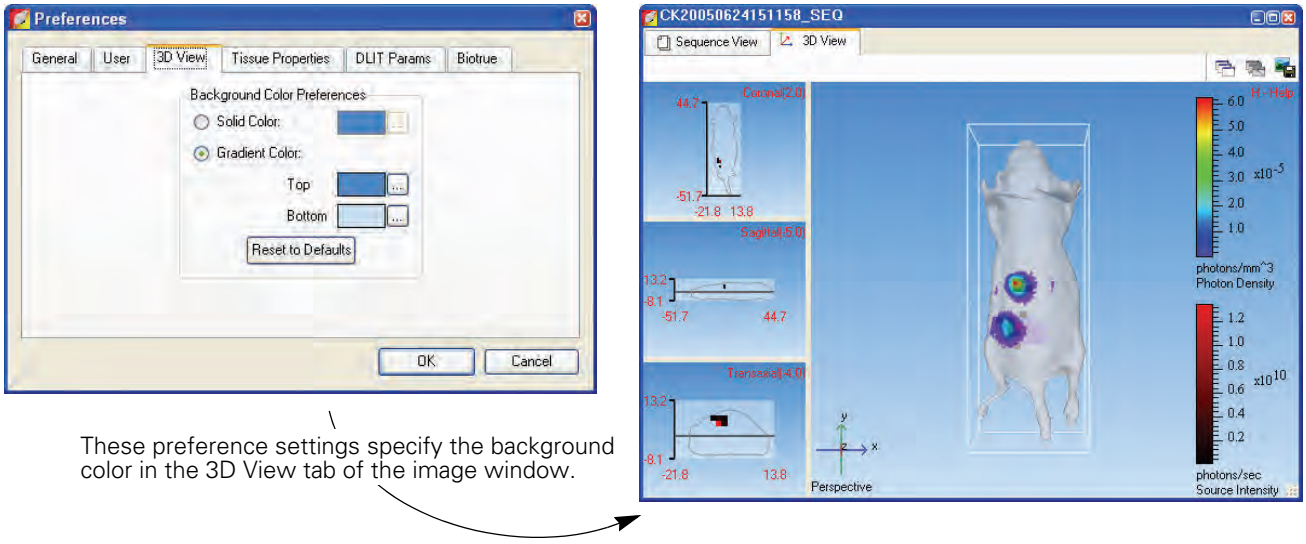
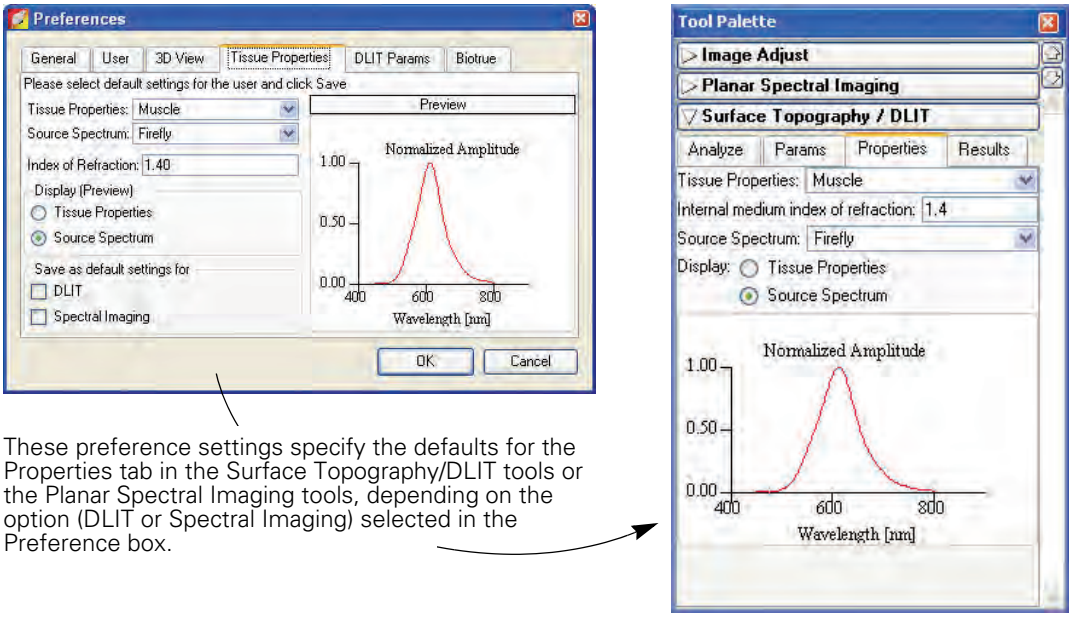


Figure H.3 Preferences, 3D View tab (left) and image window, 3D View (right)
Preference settings that affect the appearance of the 3D view in the image window.

Preferences, 3D View Tab	Description
Solid Color	Choose this option to apply a non-gradient background color to the 3D view in the image window. To select a different color or define a custom color, click the ... button.
Gradient Color	Choose this option to apply a gradient background color to the 3D view in the image window.
Top	To select a different color or define a custom color for the top of the window, click the ... button.
Bottom	To select a different color or define a custom color for the bottom of the window, click the ... button.
Reset to Defaults	Click to restore the default settings.

Tissue Properties



These preference settings specify the defaults for the Properties tab in the Surface Topography/DLIT tools or the Planar Spectral Imaging tools, depending on the option (DLIT or Spectral Imaging) selected in the Preference box.

Figure H.4 Preferences, Tissue Properties tab (left) and Surface topography/DLIT tools, Properties tab (right)

Preferences, Tissue Properties Tab	Description
Tissue Properties	Choose a default tissue type that is most representative of the area of interest.
Source Spectrum	Choose the default luminescent source.
Index of Refraction	The software automatically sets the internal medium index of refraction based on the selection in the Tissue Properties list
Display (Preview)	
Tissue Properties	Choose this option to display a graph of the absorption coefficient (μ_a), effective attenuation coefficient (μ_{eff}), and reduced scattering coefficient (μ'_s or μsp).
Source Spectrum	Choose this option to display the source spectrum.
Save as default settings for	
DLIT	Choose this option if the settings are for the Properties tab in the Surface Topography/DLIT tools.
Spectral Imaging	Choose this option if the settings are for Properties tab in the Planar Spectral Imaging tools.

DLIT Parameters

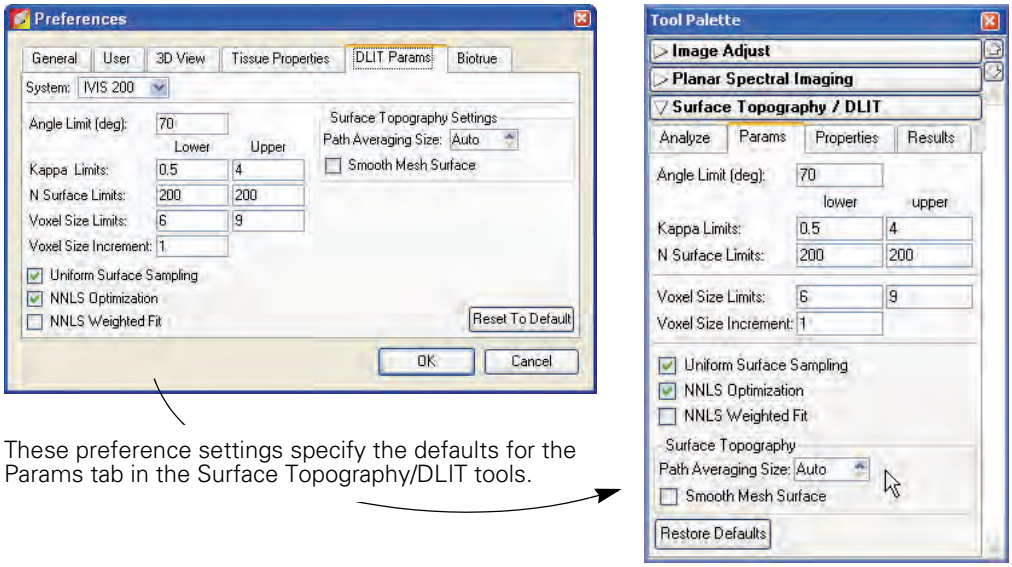


Figure H.5 Preferences, DLIT Params tab (left) and Surface topography DLIT tools, Params tab (right)

Preferences, DLIT Params Tab	Description
System	Select the IVIS Imaging System (200 Series or 3D Series) from the drop-down list.
Angle Limit (deg)	The angle between the object surface normal and the optical axis. For more details, see page 195.
Kappa Limits	Kappa (κ) is a parameter that is searched during a reconstruction to determine the best fit to the image data. Small values of kappa tend to favor deeper sources, while large values favor more shallow sources. For more details, see page 196.
N Surface Limits	The maximum number of surface intensity points to use in the reconstruction at a given wavelength. The range is 200 to 800 and the default is 200. The time required for reconstruction is shortest for smaller values of N (for example, 200). However, a large N value may give a more accurate result because more data are included in the fit.
Voxel Size Limits	Voxels are the small cubes of space inside a subject, each of which contains a light source (much like a pixel in a 2D image). The DLIT reconstruction begins with large voxels, specified by the voxel size limit (the length of a side of the voxel cube in mm). At each iteration, the algorithm reduces the size of the voxel by a factor of two until the optimum solution is found. The voxel size limits are a minimum of five and a maximum of 10. The default range is set to 6-9 mm. A larger range of voxel limits ensures a more reliable solution, but requires more computational time. The default range of 6-9 is usually adequate to determine the optimum solution.
Voxel Size Increment	This is the step increment in voxel size, stepping from the minimum voxel size limit to the maximum voxel size limit. For example, if the voxel size limit ranges from 6-9 mm, a voxel size increment = 1 gives four starting voxel sizes (6, 7, 8, and 9 mm). The default increment of 1 mm is usually adequate, however smaller increments can be used if you want to sample finer voxel sizes. Smaller increments will significantly increase the time required for reconstruction.
Uniform Surface Sampling	If this option is chosen, the surface data for each wavelength will be sampled spatially uniformly on the signal area. If this option is not chosen, the maximum 'N surface elements' will be sampled for the data. This means that the N brightest surface elements will be used as data in the reconstruction. Typically, non-uniform sampling is recommended if there is a single bright source, while uniform sampling is preferred if there are several scattered sources.

Preferences, DLIT Params Tab	Description
NNLS Optimization	If NNLS optimization option is not chosen, the software uses a linear programming algorithm to seek the solution (Simplex solution). If this option is chosen, the software also applies a non-negative least squares optimization algorithm at each iteration to provide a better solution for source power. The Simplex solution is more robust, but tends to underestimate the source flux in each voxel. Therefore, the NNLS option is recommended.
NNLS Weighted Fit	Choose this option to weight the wavelength data proportionally to its intensity in the NNLS reconstruction. This option is especially useful if the intensity of longer wavelength data is orders of magnitude greater than the intensity of shorter wavelength data.

Biotrue Preferences

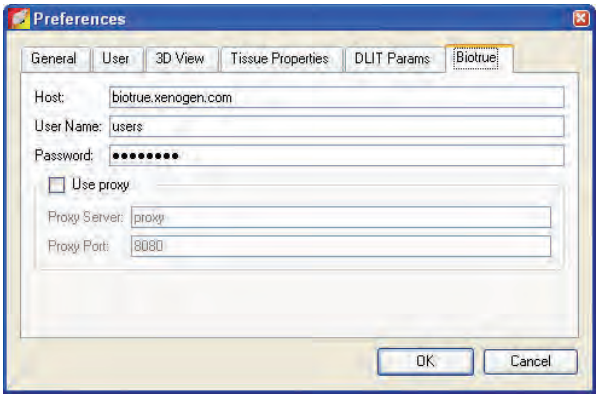


Figure H.6 Preferences, Biotrue tab
These preference settings specify how the workstation connects to the optional Biotrue Collaborative Data Management System.

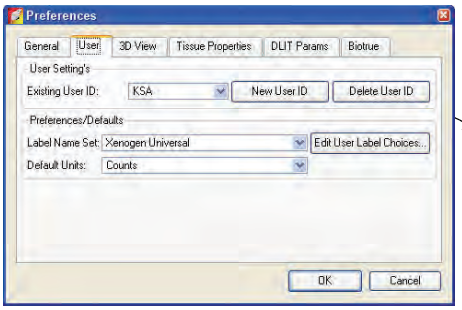
Preferences, Biotrue Tab	Description
Host	Location of the server on the network.
User Name	Your user name.
Password	Your password.
Use Proxy	Choose this option if you connect to the Internet through a proxy server.
Proxy Server	The name of the proxy server.
Proxy Port	The port used by the proxy server.

NOTE

If you use a proxy server, check with your system administrator for the appropriate proxy server name and port information.

H.2 Managing User IDs

The settings specified in the Preferences box are associated with the user ID selected at the start of a new session. The settings are applied when this user ID is selected.



To add a new user ID:

1. In the Preferences box, click **New User ID**.

2. In the dialog box that appears, enter a new user ID and click **OK**.
   - The user ID is added to the system.

To delete a user ID:

1. Select the ID that you want to delete from the Known User ID drop-down list.
2. Click **Delete User ID**.

Figure H.7 Adding or removing a user ID from the system

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